Structure/function analysis and hypoxic regulation of STREX variant BK channels

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Declaration

I hereby declare that composition of this thesis and work described herein has been carried out solely by me, except where otherwise explicitly stated, at the Centre for Integrative Physiology, University of Edinburgh. No part of this thesis has been submitted for any other degree or qualification.

Fozia Saleem

March 2008

خودی کو کر بلند اتنا کہ ہر تقدیر سے پہلے
خدا بندے سے خود پوچھے بتا تیری رضا کیا ہے

"Exalt thyself to such great heights,
That God Himself is compelled to ask you to be the master
of your own destiny"

- **Muhammad Allama Iqbal** (*Asrar-i-Khudi 1915)*

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Abstract

Large conductance voltage and calcium-activated potassium (BK) channels link electrical excitability with cellular signalling. A single gene (KCNMA1 or slo) encodes the α subunits that form the native BK channel pore. Alternative pre-mRNA splicing of the α subunits determines the channel phenotype, including its intrinsic voltage dependence and calcium sensitivity thereby providing a mechanism to generate functional diversity of BK channels.

To date the identification and functional characterization of BK channel splice variants depends on the use of low throughput patch clamp electrophysiology. The STREX (STRess controlled EXon) splice variant contains a 59 amino acid insert that enhances BK channel calcium sensitivity and also confers high sensitivity to reduced oxygen tension (hypoxia). Hypoxia sensitivity of STREX has previously been shown to be dependent upon a cysteine motif (-CSC-) in the STREX insert that is also within a putative phosphorylation motif. This thesis thus had three main aims: 1) to develop a high throughput assay to allow discrimination of different BK channel splice variants and their modulation 2) to characterise the role of cysteine residues within the STREX exon that may determine hypoxia sensitivity of the channel , and finally 3) to examine the modulation of the hypoxia response by intracellular ATP.

A high throughput screening assay for BK channels was successfully developed using the voltage-sensitive fluorescent membrane potential dye (FMP, Molecular Probes) and the automated fluid-handling fluorimeter FlexStation® II, utilising the calcium ionophore ionomycin to allow calcium influx to activate the channel. The assay allowed discrimination between

distinct BK channel splice variants, based on their different calcium sensitivities (STREX > e22 (29 amino acid insert variant) > ZERO ("null" insertless variant)), as well as examination of the pharmacological profile of the variants. While the BK channel inhibitor paxilline had a similar potency on all three variants verruculogen displayed a significantly greater potency on the ZERO and e22 variants compared to STREX.

A site-directed mutagenesis strategy combined with single channel patch clamp recording in excised inside-out patches was used to examine the contribution of all cysteine residues within the STREX insert on hypoxia sensitivity. Out of all the cysteine residues (C12, C13, C16, C23, C25 and C51) in the STREX insert; mutation of the C23 site alone completely abolished the response to hypoxia. In the absence of intracellular ATP, hypoxia caused a robust decrease in STREX channel activity, whilst in the presence of intracellular ATP a population of channels that responded with an increase in activity in response to hypoxia was observed.

As the cysteine rich hypoxia sensing motif in STREX lies within a putative phosphorylation motif (RxCS) a site-directed mutagenesis approach was used to mutate residues potentially involved in phosphorylation of the channel at this site, in an attempt to identify if the ATP modulation was via a phosphorylation dependent or independent mechanism. Mutation of the key R and S amino acid residues in the motif to alanine prevented the hypoxic inhibitory response, suggesting that the intact RxCS site in STREX is required for the response to hypoxia. However, neither the phospho null (STREX-S24A) nor phosphomimetic STREX-S24E mutant showed any significant changes in channel behaviour under hypoxia, suggesting that

phosphorylation at this site does not mediate the action of ATP and that the effects of mutation are a result of structural changes. In the presence of a non-hydrolysable analogue of ATP, AMP-PNP, the channel response to hypoxia was similar to that observed with ATP implying that ATP may mediate its effect via a phosphorylation independent mechanism.

During the course of these experiments the monovalent ion lithium (Li^+) was observed to robustly block the response of STREX channels to hypoxia. Li^+ shares physicochemical properties with the divalent magnesium (Mg^{2+}) ion hence the role of Mg^{2+} in the STREX hypoxia response was also investigated. Mg^{2+} modulated the response in a similar fashion to ATP. In addition it was seen that Li^+ and Mg^{2+} decreased the single channel conductance of STREX and ZERO variants, Mg^{2+} also increased STREX channel open probability. However, while Li^+ and Mg^{2+} may share similar mechanisms/sites to modulate channel conductance the differential effect of Li^+ and Mg^{2+} on the hypoxia response suggest Li^+ acts via a site/mechanism distinct from that of Mg^{2+}.

Taken together, these data suggest that alternative pre-mRNA splicing is an important determinant of BK channel pharmacology and that cysteine and other residues within the STREX insert play a key role in function of the channel and its response to hypoxia.

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Chapter One: Large- conductance calcium- and voltage- activated potassium (BK) channels

1.1 Introduction

Calcium-activated potassium channels are widely expressed throughout tissues of multicellular organisms (Sah, 1996; Gribkoff *et al.*, 2001a) and can be divided into three broad groups (Small, Intermediate and Big conductance, abbreviated SK, IK and BK respectively) according to primary sequence, single-channel conductance, calcium- and voltage- dependence and pharmacology. Large conductance calcium- and voltage- activated potassium channels, hereafter referred to as BK channels, are broadly distributed in many excitable and nonexcitable tissues including neurons (Wanner *et al.*, 1999), smooth muscle (Schubert & Nelson, 2001) and epithelia (Kwon & Guggino, 2004). They perform critical, diverse physiological roles such as repolarisation and hyperpolarisation of membrane potential following action potentials, regulation of neurotransmitter release, vascular tone, endocrine hormone release and hair cell frequency tuning (Toro *et al.*, 1998). Their activity may be controlled by changes in transmembrane voltage, intracellular calcium (Ca^{2+}) concentration (Toro *et al.*, 1998), the effects of phosphorylation (Levitan, 1994) and other secondary messengers (Weiger *et al.*, 1998; Brodie *et al.*, 2007).

BK channels (also known as Maxi-K, BK_{Ca} or *slo* channels) are characterised by a single channel conductance of ~250 pS in symmetrical potassium gradients (Farley & Rudy, 1988) and ~120 pS in physiological potassium gradients (Bielefeldt *et al.*, 1992). They can be independently or synergistically activated by calcium and voltage, causing an efflux of potassium ions (K^+) leading to hyperpolarisation of the membrane potential (Vergara *et al.*, 1998; Salkoff *et al.*, 2006), thus controlling cellular excitability (Meera *et al.*, 1997) and enabling a tight control of cellular processes such as calcium influx. BK channels are often found localised with voltage dependent calcium channels (Robitaille *et al.*, 1993; Grunnet & Kaufmann, 2004; Loane *et al.*, 2007), and are considered negative feedback regulators of voltage dependent calcium influx. There is accumulating evidence to suggest that BK channel modulation and signalling involves close associations with a variety of protein partners (Lu *et al.*, 2006). Consequently BK channels are powerful modulators of cellular excitability as they couple intracellular signalling with membrane potential.

1.2 Structure and function of BK channels

A landmark study (Atkinson *et al.*, 1991) revealed the first structural component encoding a calcium-activated potassium channel. Mutation of the Drosophila slowpoke (*slo*) locus specifically abolished a calcium-activated potassium current in muscles and neurons and provided an opportunity for cloning and molecular characterization of BK channels. The cDNA sequence of *slo*, known as *dSlo* then allowed for the functional expression of the Drosophila BK channel (Adelman *et al.*, 1992). The primary sequencing of BK channels showed that it belonged to the voltage-gated potassium superfamily (Jan & Jan, 1997). Interest in the role of BK channels in the years following led to many groups cloning the pore forming α subunit in tissues of interest in different mammalian species, (Butler *et al.*, 1993; Knaus *et al.*, 1995), including human (Dworetzky *et al.*, 1994; Tseng-Crank *et al.*, 1994).

The α subunit of BK channels is a ~125 kDa polypeptide and assembles as a tetramer to form the functional channel pore (Shen *et al.*, 1994), which is the minimal molecular requirement for assembly of functional channels. The α subunit can be separated into two distinct parts (Figure 1.1): a 'core' region, which contains a characteristic S0-S6 transmembrane domain structure,

resembling the α subunit of voltage-gated potassium channels (Wang *et al.*, 2003) and additionally a large intracellular C-terminus 'tail' region (S7-S10) that accounts for two thirds of the protein and includes the calcium sensor (Toro *et al.*, 1998).

1.1.1. BK channel "core" and "pore" region

Voltage-gated potassium channels have 6 transmembrane domains and an intracellular cytoplasmic N- terminus that is required for assembly of channel tetramers (Strang *et al.*, 2001). In addition this region is required for modulatory associations with voltage-gated potassium channel interacting proteins (KvChIPs) (Bahring *et al.*, 2001). BK channels belong to the 6 transmembrane voltage-gated potassium channel family and much of the transmembrane structure of the BK channel is homologous. However additionally they possess a seventh transmembrane domain (S0) that forms the extracellular N-terminus, a property unique to the BK channel, and a large intracellular C-terminus (Figure 1.1) (Latorre & Brauchi, 2006). Studies using epitope-tagged constructs showed that the N-terminus is accessible for immunolabelling in nonpermeabilised cells, demonstrating that it is exoplasmic (Meera *et al.*, 1997).

Figure 1.1
Key structural features of the BK channel

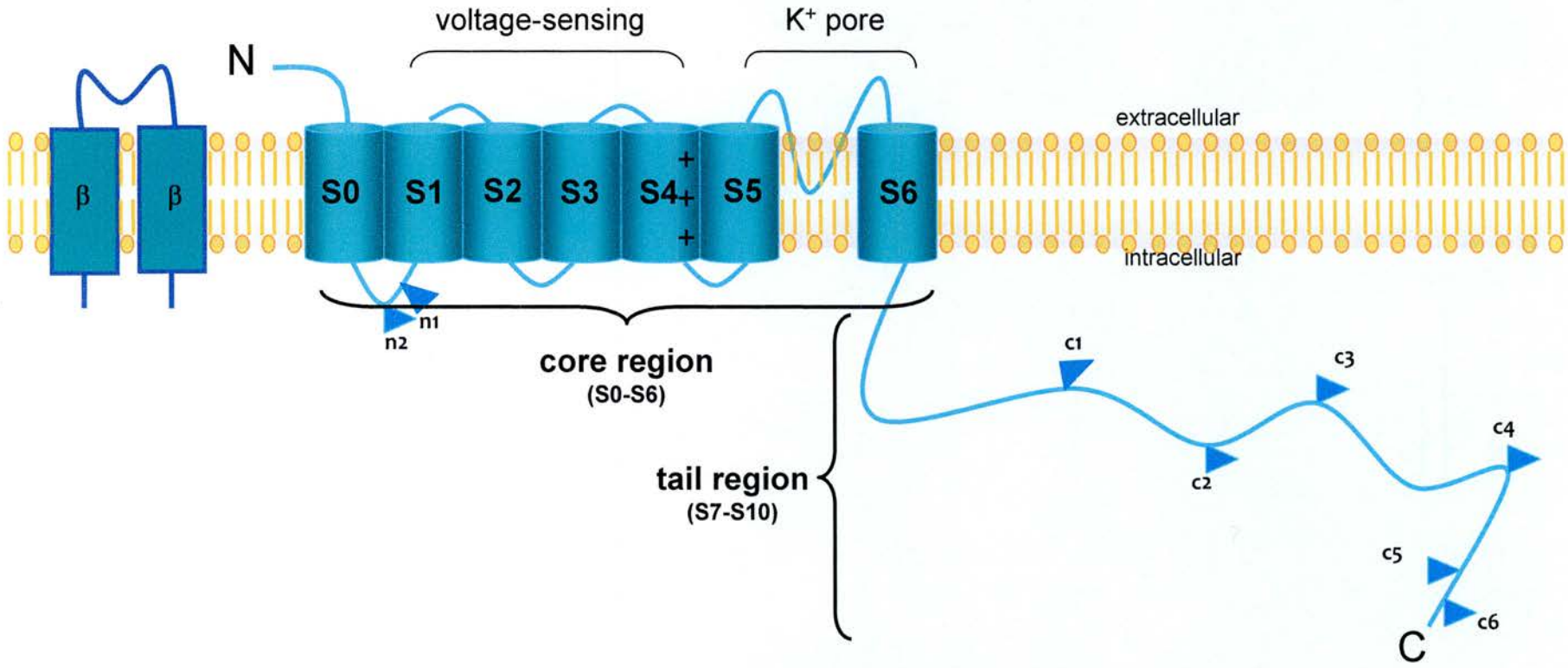


Figure 1.1 Showing transmembrane topology of BK channel, with extracellular N-terminus, 7 transmembrane segments (S0-S6), intracellular C-terminus (S7-S10), voltage - sensing regions, pore-forming region, sites of alternative splicing (n1-n2, c1-c6) and optional β-subunit (not to scale).

In the BK channel, the S0 N-terminal region is specifically required for interaction with accessory β subunits. Studies of chimeric proteins revealed that whilst the drosophila BK channel does not interact with the mammalian BK channel β subunit, replacing the S0 region with that of the human BK channel then enables such regulation (Wallner *et al.*, 1996). Interestingly although truncation of the N-terminus abolishes the association of the β1 subunit with the α subunit, but does not affect the β2 subunit interaction (Morrow *et al.*, 2006). It was shown that functional interaction of α and β1 subunits required the N-terminal tail but physical association required the S1, S2 and S3 domains of the BK channel α subunit. A recent study revealed that S0 mutants cause a rightward shift in the conductance-voltage relationship of the channel consistent with decreases in voltage sensor equilibrium, thus changing the gating kinetics of the channel (Koval *et al.*, 2007). Computational modelling of the mutants suggested that the S0 domain may make functional contact with other transmembrane regions of the BK channel hence modulating the equilibrium between resting and active states of the channel's voltage sensor.

The S4 region of the BK channel sequence contains a repeating sequence of arginines in every third position, (207, 210 and 213) and a glutamate at 219; this composition of positively charged amino acids confers the voltage sensitivity

(Papazian *et al.*, 1991; Toro *et al.*, 1998). In addition to the seven transmembrane domains known as S0-S6, BK channel α subunits have a pore region and a unique long intracellular C-terminus that has four additional hydrophobic regions and six alternative splicing sites in mammals (Figure 1.1).

The pore region (P-loop), which is assigned to the loop between the S5 and S6 region has a highly conserved TVGYG (threonine-valine-glycine-tyrosine-glycine) amino acid sequence (Heginbotham *et al.*, 1994) that is important for potassium ion selection and forms the selectivity filter of the channel pore. The P-loop is conserved across voltage-gated potassium channel families (MacKinnon, 2003). It is proposed, from structures determined by X-ray crystallography of the bacterial voltage-dependent potassuim channel (KcsA), that upon BK channel α subunit tetramer formation, the four identical subunits create an "inverted teepee" which cradles the selectivity filter of the pore in it's outer end and contains two closely placed K^+ ions that allows rapid conduction (Doyle *et al.*, 1998; Jiang *et al.*, 2001). This structure is also homologous to the pore-forming region in sodium, calcium and glutamate channels (Zhorov & Tikhonov, 2004). However use of quarternary ammonium blocking studies has revealed that in spite of similarities in assembly and structure with other

channels, BK channels have a faster rate of blocking and unblocking (Li & Aldrich, 2004)

BK channels have a similar selectivity filter (Glycine-Tyrosine-Glycine/GYG) for potassium conductance as most other lower conducting potassium channels (Heginbotham *et al.*, 1994), so the question still arises as to why they have larger conductance? Investigation of the charge of amino acid residues in the BK channel has revealed the presence of eight negatively charged glutamate residues at the entrance of the inner vestibule which are absent in other potassium channels (Brelidze *et al.*, 2003). This ring of eight negative charges arises from two charges per subunit (E321 and E324). Decreasing the charge from -8 to +8 in this "ring of charge" (Figure 1.2) causes a progressive decrease in single channel conductance. This effect on conductance is abolished at high intracellular potassium, indicating the role of an electrostatic mechanism conferred by the "ring of charge". Increasing the potassium concentration in the vestibule of the channel provides a ready source of potassium hence increasing the conductance of the channel. The "ring of charge" was seen to have little effect on inward currents whilst doubling the outward single channel conductance current. This is of particular significance as under physiological conditions currents through BK channels are outward, thereby reducing the

metabolic cost of producing channels, as a smaller number of channels produce a sufficient ionic response. Thus without this "ring of charge" BK channels become inwardly rectifying channels. Recent data has also suggested that the ring of negative charge facilitates voltage-dependent blockade of BK channels by intracellular magnesium ions (Mg^{2+}) and naturally occurring polyamines through a preferential electrostatic attraction of Mg^{2+} and naturally occurring polyamine over K^+ (Zhang *et al.*, 2006).

1.1.2. BK channel C-terminus "tail" region

The intracellular C-terminus from the S7-S10 linker region, known as the 'tail', can be expressed as a separable domain, and is important for co-expression with the rest of the channel ('the core') to produce functional channels, as neither parts alone are thought to form functional channels (Orio *et al.*, 2002; Schmalhofer *et al.*, 2005), however this is controversial (Piskorowski & Aldrich, 2002).

Two probable regulators of potassium conductance (RCK) domain structures have been determined in the BK channel tail region: one in isolation from the *Escherichia coli (E.coli)* channel, Kch (Jiang *et al.*, 2001), and another in conjunction with its pore-forming domain from the *Methanobacterium thermoautotrophicum*

channel, MthK (Jiang *et al.*, 2002a). Thereby accounting for eight RCK domains in the functional tetrameric channel (Figure 1.2), two domains per α subunit (RCK1 and RCK2) providing BK channels with an intracellular gating ring. RCK domains possess Rossman folds that form a hinged dimeric scaffold, in MthK four such dimers then combine to create an octameric assembly.

1.2.1.1 Regulation of potassium conductance domain 2 (RCK2)

The RCK2 domain is thought to be ≈ 100 amino acids downstream of the RCK1 structure (Lingle, 2007). However the exact location and boundary of RCK2 is unclear because of the poor sequence homology with known RCK domains. Several sites have been suggested, though there are critical discrepancies in the alignments (Roosild *et al.*, 2004), and one study suggested that the calcium bowl is located just outside the RCK2 domain (Kim *et al.*, 2006a). Conversely another study used structure-based multiple sequence alignment of the C terminus of the human BK channel and RCK domains of prokaryotic K^+ channels and CD spectroscopy to show that the RCK2 domain actually incorporates the calcium bowl (Yusifov *et al.*, 2008). It was suggested that RCK2 had a length almost identical to RCK1 (268 and 270 amino acids respectively) including a C-Lobe, a high-affinity calcium site, and maintained all of the conserved motifs and

Figure 1.2
BK channel tetramerisation and putative drug binding sites

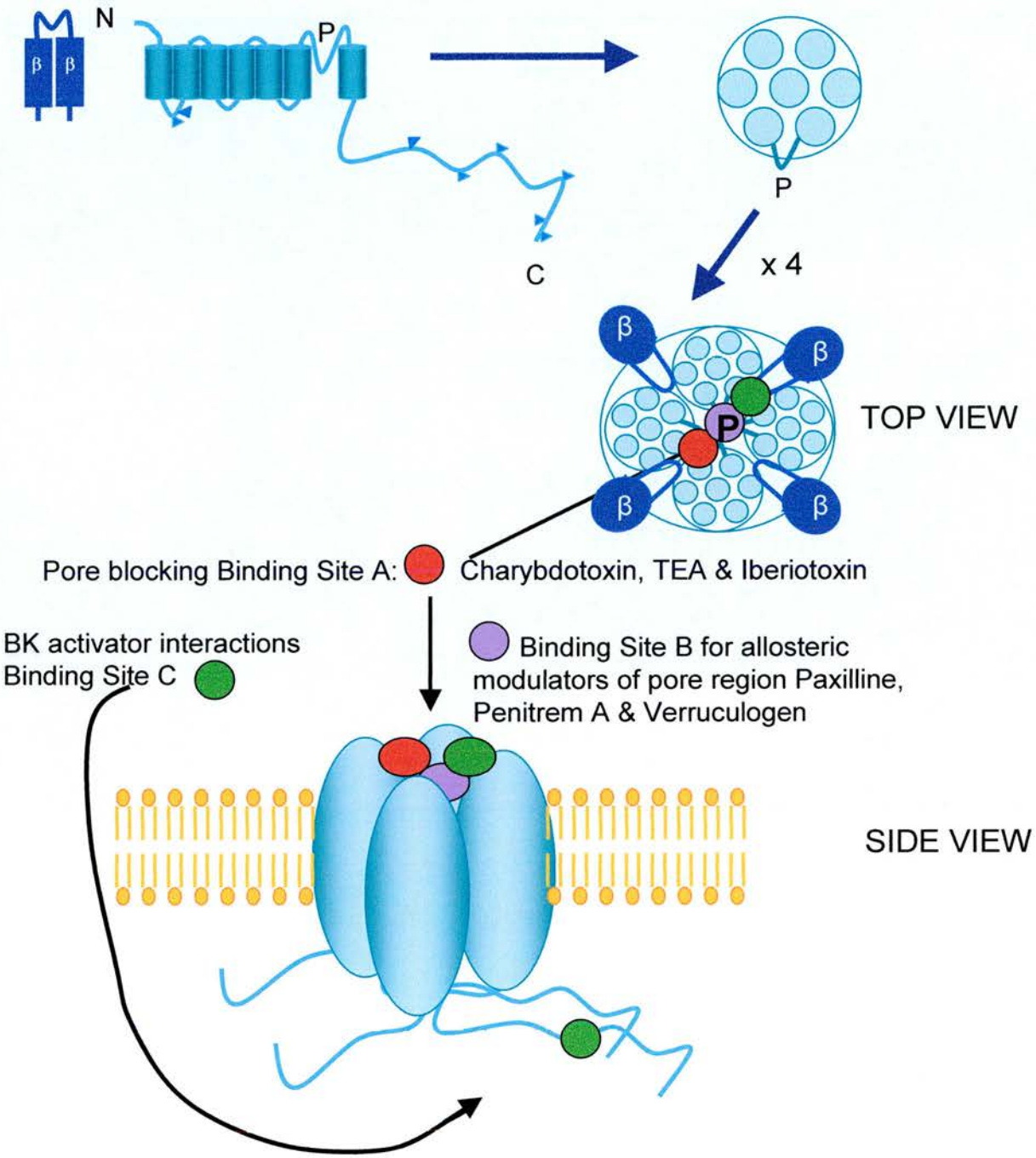


Figure 1.2 BK channel α subunit tetramer formation showing membrane topology of the channel, with extracellular N-terminus (N), 7 transmembrane segments (S0-S6), the pore region (P), intracellular C-terminus (C, S7-S10) and optional associated β subunits. Putative binding sites of BK specific drugs is indicated

residues present in RCK domains (Jiang *et al.*, 2002b) right through to the last α helix.

Binding of calcium ions to BK channels is postulated to result in the expansion of the gating ring formed by the two RCK domains, pulling on the linkers between the gating ring and results in the opening of the pore (Niu & Magleby, 2002). The energy transduction by the gating ring triggers the allosteric activation of BK (Horrigan & Aldrich, 2002).

Each RCK1 domain presumably is coupled to one RCK2 domain to form a dimer corresponding to the flexible interface and then another RCK2 domain to form an assembly interface. The inter and intra subunit co-operativity of BK channel α subunits was investigated, to determine whether the dimer pairs corresponding to the flexible interface arose from an RCK1 and RCK2 domain on a single BK channel α subunit, or domains on adjacent subunits (Qian *et al.*, 2006). The calcium dependence activation of single BK channels with two active RCK1 calcium sensors and two active calcium bowl sensors (associated with the putative calcium bowl/RCK2 domain) was examined by engineering two types of BK channels: ones in which both the RCK1 and RCK2 sensors were on the same

α subunit and ones where the active sensors were on different α subunits (Figure 1.2). When both calcium sensors are on the same subunit, a modest cooperativity in the calcium dependence of activation was observed. This suggested that intrasubunit cooperativity arises because two high-affinity calcium sensors acting across a flexible interface are more effective in opening the channel than when acting at separate interfaces.

Calcium regulation within the BK channel α subunit has been the subject of extensive site-directed mutagenesis studies (Schreiber *et al.*, 1999; Shi *et al.*, 2002; Xia *et al.*, 2002; Zeng *et al.*, 2005). The calcium bowl was previously thought to be largely responsible for the calcium sensitivity of BK and the current model locates the calcium bowl region within the putative regulation of potassium conductance domain 2 (RCK2) (Yusifov *et al.*, 2008) of the intracellular C-terminus tail region (Figure 1.2). It is highly conserved in all cloned BK channels and contains an aspartic acid (Asp) rich sequence motif (Glutamine-Asp-Asp-Asp-Asp-Asp-Proline-,/-QDDDDDP-). A study involving chimeric channels confirmed the localization of the calcium sensitivity to the end of the S9-S10 linker and part of the S10 hydrophobic segment (Schreiber *et al.*, 1999).

Calcium binding studies using a fusion protein consisting of Drosophila Slo1 channel residues showed that by replacing all aspartic acid residues with uncharged asparagine residues, the calcium bowl motif only accounted for ~ 56% of the calcium binding, indicating the presence of other sites responsible for the calcium binding (Bian *et al.*, 2001). Truncated BK channels lacking the intracellular C-terminus have been reported as having calcium sensitivity similar to that of the wild- type channel (Piskorowski & Aldrich, 2002), providing further evidence for calcium binding sites outside the calcium bowl.

A second high-affinity calcium binding region has also been identified ≈ 400 amino acids upstream of the calcium bowl within the RCK1 domain. Neutralization of a methionine (513) (Bao *et al.*, 2002) or two aspartate (D362/D367) (Xia *et al.*, 2002; Zeng *et al.*, 2005) residues impaired the calcium sensing of this domain. The presence of distinct high and low affinity divalent cation binding sites (E374, Q397 and E399) have also been identified within the RCK1 domain and thought to mediate the overall magnesium sensitivity of BK channels (Zeng *et al.*, 2005; Hu *et al.*, 2006a; Yang *et al.*, 2006). Thus the location and molecular characterisation of specific calcium binding sites within the BK channel requires further resolution.

1.2.1.2 Regulation of potassium conductance domain 1 (RCK1)

Tetrameric assembly of BK channels is thought to be dependent on the presence of association domains (ADs). Functional assays investigating putative AD's in *Xenopus* oocytes led to the identification of a hydrophobic linker region (L6) downstream of the pore-forming domain between the hydrophobic S6 and S7 (Quirk & Reinhart, 2001). It has been shown to self- associate to form tetramers, and is the only hydrophilic region of the channel that is able to do so. This novel tetramerization domain, referred to as BK-T1, is thought to promote the assembly of BK channel α subunit monomers into functional BK channels. However the RCK1 domain shows overlap with the BK-T1 containing L6 region. Although sequence homology between the two is just 20%, the residues conserved in the prokaryotic RCK sequence are also conserved in the eukaryotic BK channel.

The importance of the RCK and the BK-T1 domains is brought into question as a truncated BK channel ($BK_{1\text{-}323}$) lacking the entire intracellular C-terminus was shown to form channels with the same characteristics as full-length BK channel in *Xenopus* oocytes (Piskorowski & Aldrich, 2002). In order to clarify the role of the C-terminus in BK channel structure and function, the effect of truncating the

channel after the S6 transmembrane domain was investigated (Schmalhofer *et al.*, 2005). Contrary to previous data reported (Piskorowski & Aldrich, 2002) the BK_{1-323} did not show any tetramerization, cell surface expression or channel function under various different conditions. BK_{1-343} and BK_{1-441} complexes were able to form tetramers, associate with β1 subunits and display cell surface expression. In addition their pharmacology, as determined by radioligand and fluorescent binding studies, was indistinguishable from wildtype BK channels but they did not form functional channels. The BK_{1-651} complex underwent tetramerization and correctly associated with the β1 subunit but did not show cell surface expression and was internally retained. Co-expression of the BK_{1-651} complex with the C-terminus ($BK_{651-1113}$) rescued cell surface expression and function, though BK_{1-343} and BK_{1-441} functions could not be rescued with co-expression of their respective C-terminal regions. The results presented in this study (Schmalhofer *et al.*, 2005) demonstrated that the S0-S6 linker domain of the BK channel, comprising the first 343 residues of the protein, was sufficient for channel tetramerization, interaction with the β1 subunit and cell surface expression. Additionally it was shown that the two RCK domains of the BK channel, containing residues 344-1113, were not necessary for these functions, but do play a critical role in channel function. Interestingly though when the core and tail domain are expressed as separate peptides in *Xenopus* oocytes functional

channels are formed that are indistinguishable from wildtype BK (Wei *et al.*, 1994).

1.3 Regulation of BK channels

Ion channels are dynamic proteins that can switch between different open and closed states. The open conformation is when ion flow is permitted through the channel and the closed being when it is not. Equilibrium exists between these two states that determines the amount of current which flows across the membrane, this equilibrium is influenced by factors such as membrane voltage, association with ligands such as neurotransmitters, intracellular messengers such as calcium or cyclic nucleotides, covalent modification of the channel by phosphorylation or by pharmacologically active openers or blockers.

The BK channel pore-forming α subunit is encoded only by a single gene, KCNMA1, and the amino acid sequence is highly conserved across mammalian species, with > 97% amino acid identity, unlike other voltage- gated ion channels which have a variety of isoforms arising from multiple genes. Remarkably although several functionally distinct BK channels have been reported, this can

be attributed to the modulation of BK channel properties by a variety of factors, such as assembly with four accessory β subunits (Orio *et al.*, 2002; Torres *et al.*, 2007), extensive pre- mRNA splicing of the α-subunit (Shipston, 2001) and metabolic and posttranslational regulation (Schubert & Nelson, 2001). As a result, altered calcium and voltage sensitivities (Xie & McCobb, 1998), shifts in regulation by protein kinases (Tian *et al.*, 2001a), changes in gating kinetics and pharmacological properties (Toro *et al.*, 1998; Orio *et al.*, 2002) and significant changes in subcellular localisation of the BK channel α subunit (Zarei *et al.*, 2001) are all possible.

1.1.3. Regulatory β subunits

The N-terminal S0 transmembrane domain of the BK channel pore forming α subunit is required for association with auxiliary β subunits (Figure 1.1). This interaction has been shown to be a significant regulator of channel function, with expression of particular β subunits being limited to specific tissues (Jiang *et al.*, 1999). Association of the α subunit with the different β subunits alters the pharmacological properties (Dworetzky *et al.*, 1994; McManus *et al.*, 1995; Meera *et al.*, 2000), voltage dependence (Wallner *et al.*, 1995; Ramanathan *et al.*, 1999),

apparent calcium sensitivity (Ramanathan *et al.*, 1999) and kinetics of assembled channels (Nimigean & Magleby, 1999; Wallner *et al.*, 1999).

The first β subunit was identified as a glycosylated 31 kDa peptide of 191 amino acids, associated with BK channels in smooth muscle membrane preparation with high affinity for charybdotoxin, a peptide blocker of BK channels (McManus *et al.*, 1995). The β subunit consists of two putative transmembrane domains connected by an extracellular loop that has four conserved cysteine residues (Hanner *et al.*, 1998). This allows disulphide linkage which induces a change in BK channel α subunit conformation hence promotes charybdotoxin binding. It was later established that this β subunit was only one of a family of auxiliary β subunits from mammals (β1-β4) (Knaus *et al.*, 1994a; Knaus *et al.*, 1994b; Wallner *et al.*, 1999; Brenner *et al.*, 2000b; Meera *et al.*, 2000) and was thus renamed β1 (Orio *et al.*, 2002).

β1 affects the gating and apparent calcium sensitivity of the α subunit (Nimigean & Magleby, 1999; Bao & Cox, 2005), is expressed primarily in smooth muscle and hair cells and has many physiological implications. For example, downregulation of the β1 subunit, is associated with elevated blood pressure in

rats (Amberg & Santana, 2003) and targeted deletion of the gene leads to a disruption of vasoregulation (Brenner *et al.*, 2000b) and of phasic contractions of the urinary bladder (Petkov *et al.*, 2001), consistent with the prominent expression of BK channels in smooth muscle. The occurrence of a gain-of-function mutation of the β1 subunit in humans was found to be protective against diastolic hypertension (Fernandez-Fernandez *et al.*, 2004), providing further evidence for a role for this particular β subunit in control of vascular resistance (Nelson & Bonev, 2004). It has also been proposed that the β1 subunit could be responsible for the high sensitivity to intracellular calcium that native BK channels show in hair cells (Fettiplace & Fuchs, 1999).

Screening of the human expressed sequence tag (EST) databases led to the identification of another β subunit, which was selectively expressed in chromaffin cells and brain and was named β2 (Xia *et al.*, 1999). Calcium sensitivity and gating kinetics of BK channels formed by α and β2 subunits are similar to those of channels formed by α and β1 subunits; however in contrast to β1 the β2 subunit confers low affinity for charybdotoxin and promotes a fast inactivation mechanism of the BK channel (Wallner *et al.*, 1999).

Thereafter β3 and β4 were identified from human EST databases and subsequently cloned almost simultaneously (Behrens *et al.*, 2000; Brenner *et al.*, 2000a; Xia *et al.*, 2000). β3 was found to be structurally similar to β1 and β2. β3 was expressed mainly in the testis, pancreas and spleen and has four known splice variants at the N-terminus (Xia *et al.*, 2000). Three of these variants confer fast inactivation properties to the channel, whilst none alter the calcium sensitivity. The β4 subunit is expressed predominantly in the brain (Brenner *et al.*, 2000a) and has complex effects on BK channel calcium sensitivity. Furthermore, knockout of the β4 subunit leads to increased neuronal firing rates and temporal lobe seizures (Brenner *et al.*, 2005), thus indicating that β4 is an important regulator of neuronal firing. Uniquely β4 subunit channels confer insensitivity to nanomolar concentrations of charybdotoxin and iberiotoxin (Meera *et al.*, 2000). It is thought that the extracellular loop of the β4 subunit which forms a part of the toxin receptor prevents the interaction of the toxin with its binding site (Binding Site A, Figure 1.2). β subunits therefore can alter the calcium sensitivity and gating kinetics of BK channels, as well as modifying channel pharmacology and in doing so provide the functional diversity required by BK channels to mediate its many physiological roles.

Figure 1.3
BK channel C-terminus interactions

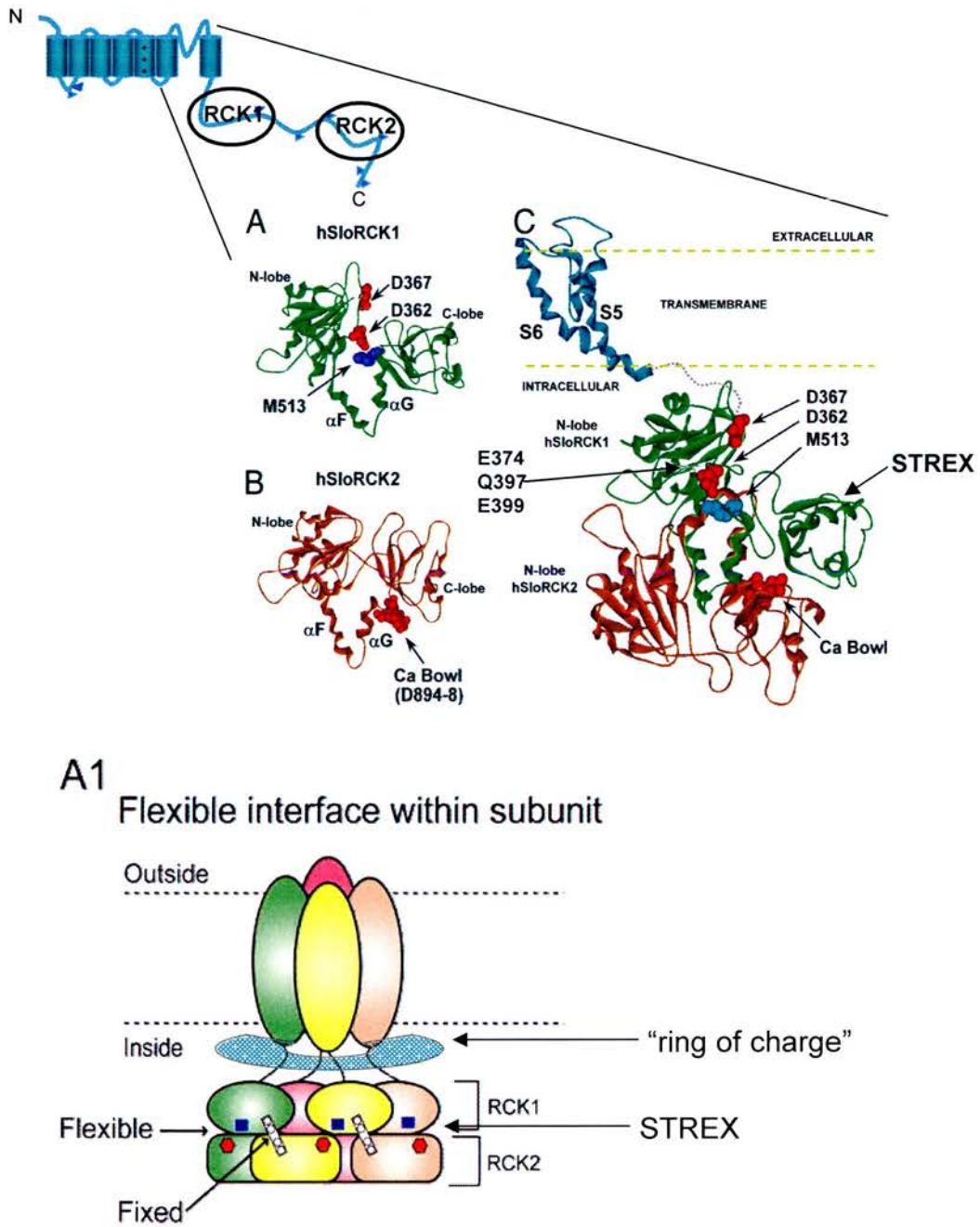


Figure 1.3 BK channel α subunit: proposed binding sites of cations and folding of RCK structure are indicated, along with putative interactions in the assembled tetramer. The potential location of the STREX insert is also indicated.

Figures adapted from Qian *et al.*, 2006 and Yusifov *et al.*, 2008

1.1.4. Alternative splicing of BK channels

Another means of generating diversity among products of a single gene is known as pre-mRNA splicing, this involves the processing of primary transcripts to remove the non-coding intronic sequences, and selection of exons for inclusion to generate mature mRNA (Figure 1.3). This process is reliant on multiple protein-protein, protein-DNA and protein-RNA interactions and frequently occurs co-transcriptionally, although this coupling is not obligatory (Kornblihtt *et al.*, 2004). Splicing takes place in the multiprotein complex known as the spliceosome, which is formed of up to 300 proteins and 5 RNAs (Jurica & Moore, 2003). Whilst already subject to multiple levels of control regarding splicing occurrence and efficiency (Fong *et al.*, 2003), additional interactions also exist that only occur during specific states, such as depolarisation, that can specifically regulate the inclusion or exclusion of certain exons (Xie & Black, 2001).

Alternative pre-mRNA splicing of α subunits plays a major role in modifying the physiological properties of the BK channel. It can modify the protein encoding capacity of the mRNAs by including or excluding specific coding segments (known as exons) (Shipston, 2001). Cloned BK channel splice variants exhibit distinct phenotypes in heterologous expression systems (Lagrutta *et al.*, 1994; Tseng-Crank *et al.*, 1994).

Figure 1.4
Overview of pre-mRNA alternative splicing

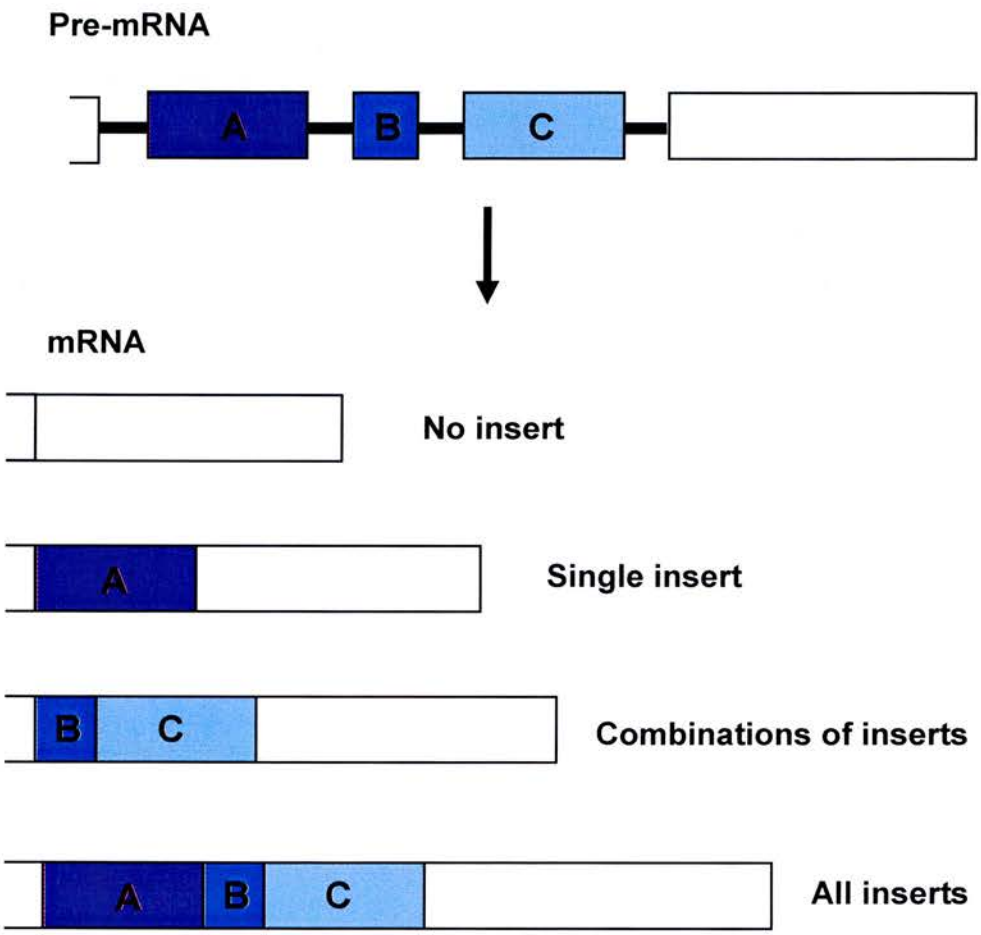


Figure 1.4 Possible fates of pre-mRNA during splicing. Alternative exons, A, B and C may be excluded from mature mRNA, included singly or in combination, or all included to form several products, increasing diversity of proteins encoded by a single gene.

Properties that can be further modified by reversible protein phosphorylation (Levitan, 1999; Tian *et al.*, 2001a). BK channel splicing is also regulated by CaMK-IV-dependent phosphorylation (Xie & Black, 2001). There are eight major sites of alternative splicing in the mammalian BK channel, two at the extracellular amino terminus and six at the intracellular C-terminus (Figure 1.1), although additional species-dependent sites are thought to exist.

Furthermore, behavioural manipulations have shown that alternative splicing can be dynamically regulated in adult mammals, enabling the fine- tuning of cellular excitability, for example in adrenal chromaffin cells, to favour passive coping responses by downregulation of sympathoadrenal function following long- term behavioural stress (McCobb *et al.*, 2003). The alternative splicing of BK channels can therefore be seen as a mechanism for adjusting cellular excitability in response to physiological requirements

Investigation of BK channel splice variants of the c2 splice site (Figure 1.4) highlighted the different intrinsic voltage and calcium sensing properties between four different variants; the cysteine rich 59 amino acid e21 ('STRess axis-regulated EXon' thus commonly known as STREX), 29 amino acid insert e22, a

three-amino acid (IYF) insert e20 and insertless null variant known as ZERO (Chen *et al.*, 2005) (these BK channel variants will hereafter be referred to as STREX, e22, IYF and ZERO channels).

The STREX variant shows increased expression in mouse adult endocrine tissues and e22 more so during embryonic stages of mouse development. The variants were differentially sensitive to phosphorylation by endogenous cAMP-dependent protein kinase; ZERO, e20, and e22 variants all underwent activation whereas surprisingly the STREX variant was inhibited. Additionally it has been shown that BK channel expression is upregulated in the murine central nervous system (CNS) during embryonic and postnatal development (MacDonald *et al.*, 2006). Although expression of the STREX variant was highest at the early embryonic stage, this was robustly downregulated with postnatal development accompanied by an increase in ZERO variant expression. This implies that exclusion of the STREX exon is under strict regulation in the developing murine CNS that may result in changes in the physiological function of the BK channel during mammalian brain development.

Figure 1.5
BK channel splice variants

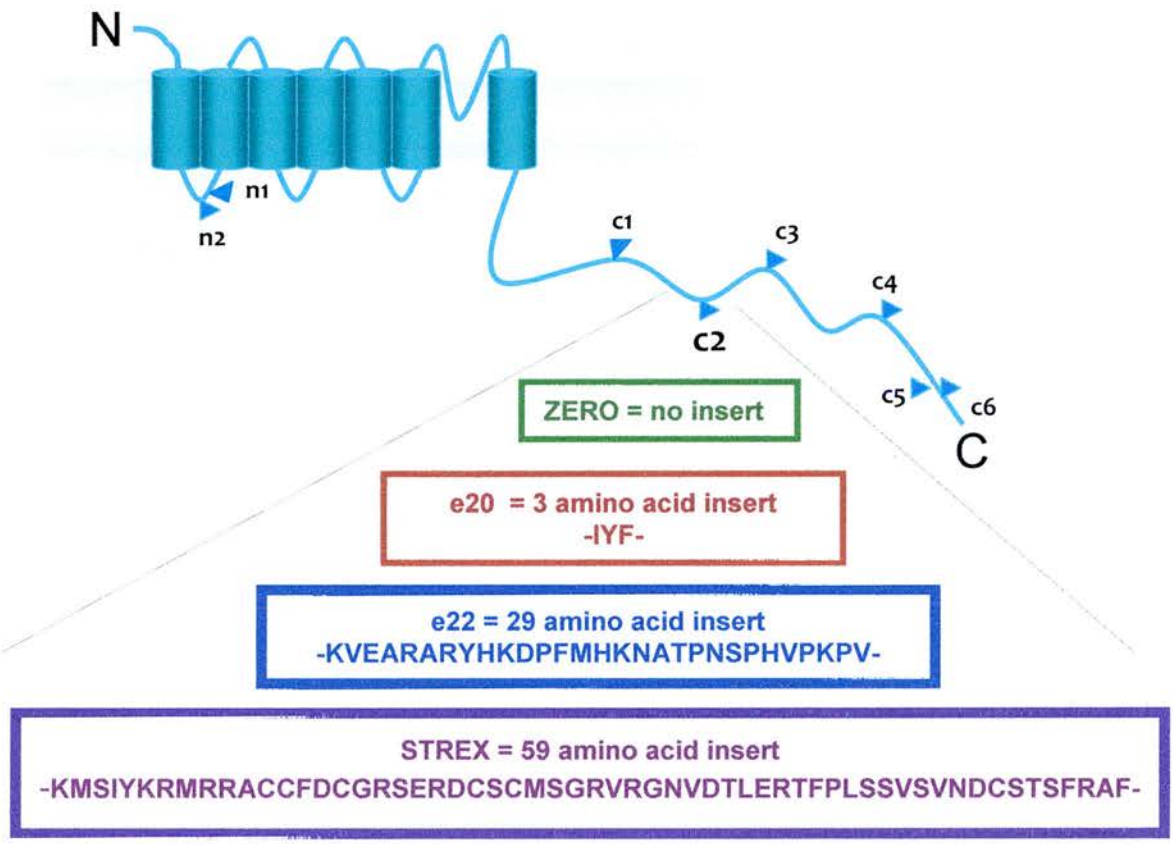


Figure 1.5 BK channel α subunit splice insert sites are indicated at the C- and N- terminus. The BK channel c2 splice variants most studied: ZERO, e20, e22 and STREX are indicated.

A large number of studies have focussed on one of the most commonly alternatively spliced variants of the BK channel, containing the STREX insert. Inclusion of this exon is influenced by perturbations of the hypothalamic-pituitary-adrenal (HPA) axis. The ratio of STREX to ZERO BK channel splice variant transcripts can be reduced following ablation of HPA axis activity by removal of the pituitary gland (hypophysectomy) (Xie & McCobb, 1998).

Inclusion of the STREX exon in BK channel α subunits confers increased sensitivity to calcium (Saito *et al.*, 1997) as well as a reversal of channel response to PKA phosphorylation; STREX channels are inhibited by PKA whereas those lacking STREX are activated (Tian *et al.*, 2001b). STREX channels also display increased activation and decreased rate of channel deactivation (Xie & McCobb, 1998). This switch in channel phenotype resembles the physiological behaviour of BK channels in the uterus where non-gravid myometrium expresses a BK channel population that is inhibited by PKA whilst during pregnancy most of the channels are positively regulated by PKA (Perez & Toro, 1994; Zhou *et al.*, 2000). Thus, STREX can be considered a signalling–transducing exon as it may switch β-adrenergic stimulation linked to PKA activity.

1.1.5. Phosphorylation of BK channels

BK channels are modulated by various different protein phosphatase and kinase signalling pathways (Chung *et al.*, 1991; Reinhart & Levitan, 1995; Schubert & Nelson, 2001; Erxleben *et al.*, 2002b; Tian *et al.*, 2004; Levitan, 2006). There are numerous possible phosphorylation sites in the BK channel α subunit, mainly in the C-terminus, that induce a diverse range of properties (Reinhart & Levitan, 1995; Nara *et al.*, 1998; Zhou *et al.*, 2001; Tian *et al.*, 2007). Phosphorylation by protein kinases such as cAMP- dependent protein kinase (PKA), protein kinase C (PKC) and cGMP-dependent protein kinase (PKG) is an important means of post-translational BK channel regulation, and is significant in modulation of BK channel calcium- and voltage- sensitivities during changes in Ca^{2+} concentration and membrane depolarisation (Schubert & Nelson, 2001).

Regulation of BK channel function via phosphorylation by endogenous cAMP-dependent protein kinase (PKA) has been shown to require a leucine zipper motif (LZ) (Tian *et al.*, 2003), which is known to be significant in ion channel assembly (Jones *et al.*, 2004) and also in the interaction of protein kinases with other ion channels (Hulme *et al.*, 2002). The stereotypical LZ domain consists of a seven residue (heptad) repeat, with hydrophobic residues at the first and fourth positions, and is critical for PKA phosphorylation, as it mediates anchoring of the

kinase to several types of ion channels by A Kinase-Anchoring proteins (AKAPs). Association of the β2-adrenergic receptor with BK channels is thought to require AKAPs, to modulate β-adrenergic regulation of membrane excitability (Liu *et al.*, 2004). In the BK channel, a highly conserved, non- canonical LZ motif exists downstream of the RCK domain, at residues 513-548 in the intracellular C-terminal tail. It is required for the PKA-mediated regulation (Tian *et al.*, 2001b) of ZERO and STREX BK channel alternative splice variants in a manner that is independent of AKAPs (Tian *et al.*, 2003). A second putative LZ motif is located at residues 816-843, and it is thought that in the C-terminus of the BK channel there exist at least two additional sites containing LZ- like heptad repeats.

It has been shown that phosphorylation-induced activation and inhibition of BK channels containing alternatively spliced α subunits is subunit-specific (Tian *et al.*, 2004). Site-directed mutagenesis was utilised to generate TEA-insensitive BK channel α subunits, mutating a tyrosine residue (Y334V) at the external mouth of the channel, which were then expressed alongside wildtype BK channel α subunits. It was found that activation by cAMP-dependent PKA phosphorylation, of a conserved C-terminal PKA consensus S899 site, required all four BK channel α subunits to be phosphorylated for the ZERO BK channel variant to be activated. In contrast PKA dependent inhibition of the STREX

variant required phosphorylation of only a single α subunit at the STREX specific PKA consensus site S3, as found by assembly of STREX and ZERO heterotetramers. This highlighted a mode of BK channel regulation by PKA phosphorylation in an "all-or-nothing" rule for activation but a "single-subunit" rule for inhibition. It can therefore be seen that there is a strong possibility that heteromeric assembly of alternatively spliced BK channel α subunits occurs *in vivo*, but that a hierarchy exists to determine the functional phenotype.

Protein kinases regulate BK channel activity in many tissues (Schubert & Nelson, 2001; Zhang *et al.*, 2004) and variable responses to the same kinase, PKA for example, can be elicited by alternative splicing of the BK α subunit. Kinase or phosphatase activity most likely forms a context-dependent means of selective activation or inhibition of a specific subset of the channel population (Widmer *et al.*, 2003). Evidence also exists for differential regulation by other cyclic nucleotide-dependent protein kinases, (Barman *et al.*, 2003; Barman *et al.*, 2004), with functional cross-activation between the signalling pathways for example, in pulmonary smooth muscle, PKC-induced relaxation is achieved by opening of BK channels via cGMP-dependent kinase. As well as the conserved phosphorylation sites in the BK channel, additional sites may be added by alternative splicing (Tian *et al.*, 2001b). Therefore it is evident that multiple

modes of regulation can exist, and as for other aspects of channel function, such as tetrameric assembly, there may be a hierarchy regarding which subunits control the phenotype of the whole channel.

It was recently demonstrated that BK channels assemble with closely associated cellular signalling proteins, including the actin-binding protein cortactin (Tian *et al.*, 2006) that was originally identified as a major src tyrosine kinase substrate. Cortactin can be phosphorylated on multiple tyrosine residues and its phosphorylation status has been implicated in modulation of actin cytoskeleton (Lua & Low, 2005). Several studies have previously investigated the tyrosine phosphorylation role in BK channels, showing that it has a diverse range of functional effects on the channel (Alioua *et al.*, 2002; Ling *et al.*, 2004). Further investigation has elucidated that the mammalian BK channel is regulated by endogenous protein tyrosine phosphatase and kinase activity that is closely associated with the channel (Tian *et al.*, 2008). This regulation is not mediated by direct phosphorylation of the BK channel α subunits themselves but by tyrosine phosphorylation of cortactin which co-assembles with BK channels at the cell membrane. This is a novel mode of BK channel regulation and follows the emerging concept that ion channels exist in the membrane as a component of a dynamic signalling protein complex, which includes the channel protein itself

associated with one or more proteins that allow regulation of channel activity. Thus phosphorylation dependent regulation of accessory proteins within the BK channel signalling complex is an important target for control of BK channel activity.

Additionally, there exist other, physiologically significant forms of post-translational regulation of BK channel activity, such as redox regulation. Methionine and cysteine oxidation have been shown to act in an opposing manner to regulate BK channel opening, and therefore may be of significance in mediating effects of reactive oxygen species on cell excitability (Tang *et al.*, 2001). Changes in oxygen tension also regulates the activity of BK channels (see Section 1.5)

1.4 Physiology of BK channels

BK channel expression is implicated in homeostasis (Sausbier *et al.*, 2005; Rieg *et al.*, 2007), development (MacDonald *et al.*, 2006) and immune function (Ahluwalia *et al.*, 2004). The expression of a range of alternative splice variants with differing functional phenotypes means that perturbation of BK channel

function can also contribute to certain pathological states (Meredith *et al.*, 2004; Weaver *et al.*, 2004; Weaver *et al.*, 2006). The first identified BK channelopathy, a human syndrome of concurrent generalized epilepsy with paroxysmal dyskinesia is caused by a missense mutation. A single A-G base mutation in the BK channel α subunit causes the aspartic acid residue at position 434 in the RCK1 domain of the channel protein to be changed to glycine (Du *et al.*, 2005). This has the effect of increasing BK channel calcium sensitivity three- to five fold, thus causing disregulation of neuronal excitability, resulting in epilepsy and dyskinesia.

BK channel α subunit knockout mice ($BK^{-/-}$), which do not express BK channel α subunits, show several symptoms of cerebellar learning deficiency, such as ataxia and inability to develop conditioned eye-blink responses to stimuli (Sausbier *et al.*, 2004). The cerebellum in the knockout mice was morphologically similar to that of the wild type, therefore implying that BK channel α subunit expression may not be required for gross morphological formation of the cerebellum but is critical for correct cerebellar function and motor learning. In addition, a decrease in spontaneous activity of $BK^{-/-}$ mice cerebellar Purkinje neurons was observed. These are responsible for generating the sole output of the cerebellar cortex and

enhanced short-term depression at the only output synapses of the cerebellar cortex, in the deep cerebellar nuclei.

In the β1 subunit knockout mice ($\beta1^{-/-}$) there was no change in body or kidney function but the K^+ excretion is decreased and mean arterial pressure was higher when compared to wildtype ($\beta1^{+/+}$) (Pluznick *et al.*, 2003; Pluznick & Sansom, 2006). Marked differences in bowel structure and colon function were also observed with β1 subunit deletion. The animals had loose faecal matter and a weaker structural integrity of colon than the wild type (Hagen *et al.*, 2003). This could be due to the altered calcium sensitivity of BK channels lacking the β1 subunit causing stronger colonic smooth muscle contractions. A recent study has also shown a decrease in sensitivity of BK channels to voltage, calcium and the specific inhibitor iberiotoxin in type-1 diabetic myocytes (Dong *et al.*, 2008). The expression of β1 subunit but not α subunit is decreased in cerebral arteries of diabetic mice. This diabetic impairment of BK channel activity is absent in the carotid artery smooth muscle cells in BK channel $\beta1^{-/-}$ mice. Hence this impairment of β1 subunit in diabetic vascular smooth muscle cells results in increased vasoconstriction and elevated blood pressure, thereby contributing to vascular diseases in type-1 diabetes.

The $BK^{-/-}$ mice progressively developed outer hair dysfunction and degeneration starting from 8 weeks even though they had a normal inner hair cell phenotype (Ruttiger *et al.*, 2004) suggesting a role for the BK α subunit in maintaining outer hair cells that receive efferent feedback from the brain. Notably though the severity of increased mean arterial pressure was greater in the BK β1 knockout mice than the BK α deficient mice, highlighting the selective role of β1 subunit in controlling vascular tone.

Coordinated BK channel activity contributes to correct maintenance of blood pressure by regulating arterial diameter (Wellman and Nelson, 2003, Standen and Quayle, 1998). The increased intracellular calcium concentration that accompanies contraction of vascular smooth muscle cells leads to elevation of calcium in the sarcoplasmic reticulum. Subsequent local release of calcium as 'calcium sparks' from the sarcoplasmic reticulum through ryanodine-sensitive calcium release channels (ryanodine receptors), causes activation of BK channels and potassium efflux (Jaggar *et al.*, 2000; Wellman & Nelson, 2003), thereby enabling feedback regulation of cell excitability during smooth muscle contraction. Disregulation of this coupling of calcium sparks to BK channel activity may be an underlying cause of impaired vascular contraction in

hypertension, and has been shown to be due to decreased expression of the BK channel β1 subunit (Amberg & Santana, 2003).

BK channels present in the inner mitochondrial membrane of cardiac myocytes have a cytoprotective role during ischemia, possibly by increasing the mitochondrial potassium influx (Xu *et al.*, 2002). A potential role for BK channel blockers is suggested in conditions such as ischemic reperfusion injury where an abnormal enhancement of BK channel activity, mediated via NOS activation, predisposes to hyperkalemia (Tricarico *et al.*, 2002).

In urinary incontinence, abnormal enhancement of muscle excitability results from the lack of functional BK channels, which maintain the balance between contraction and relaxation of the urinary bladder smooth muscle. Mice lacking the BK channel gene show a significant elevation in urination frequency (Meredith *et al.*, 2004), possibly due to the lack of β1 subunit regulation that shapes the myogenic contractions of the smooth muscle (Petkov *et al.*, 2001). Additionally β-adrenoceptor stimulation of urinary bladder smooth muscle activates the BK channel to facilitate muscle relaxation (Kobayashi *et al.*, 2000;

Petkov & Nelson, 2005), therefore providing new drug targets for treatment of unstable bladder.

Another role of the BK channel is in control of potassium secretion (Woda *et al.*, 2001). In the kidney, baseline potassium secretion is primarily through SK channels, whilst BK channels mediate flow-dependent potassium secretion. Disruption of BK channel expression may lead to inappropriate or excessive loss of potassium, therefore tight regulation of expression must be maintained to enable homeostasis. In patients with end-stage renal disease (ESRD), the potassium permeability in the colon is increased up to three times that of normal individuals (Mathialahan *et al.*, 2005), attributable to increased expression of BK channels in surface colonocytes, as well as crypt cells, where they are absent in non-ESRD individuals, and the increased potassium loss is sufficient to be physiologically significant.

Since disregulation of BK channel expression is a facilitator of pathological conditions, there is increasing evidence that certain splice variants of the channel are markers for disease. For example, the splice variant glioma BK (gBK) has been shown to be highly expressed in glioma cells, encoding a BK channel

containing a 34 amino acid insert at splice site c2 that enhances channel sensitivity to intracellular calcium concentration (Liu et al., 2002). Although gBK is also expressed in normal cortical tissues, its expression is upregulated in glioma cells. Additionally, expression of BK channels has also been shown to be positively correlated with tumour malignancy grades, therefore this novel splice variant may be a facilitator of tumorigenesis.

1.5 BK channel pharmacology

The role of BK channels in disease is therefore of significant importance and pharmacological manipulation of these channels may be of therapeutic benefit. BK channel activators stabilize the cell by increasing potassium efflux leading to hyperpolarisation, hence decreasing cell excitability potentially leading to smooth muscle relaxation. The activators comprise a large series of synthetic benzimidazolone derivatives, such as NS004 and NS1619, the biaryl amines, such as mefenamic and flufenamic acids, the biarylureas, such as NS1609, the pyridyl amines, natural modulators like dihydrosoyasaponin-1 (dehydrosoyasaponin-1; DHS-1), and flavonoids.

The activators are thought to increase BK channel activity via several mechanisms; by modulating the calcium binding site at the C-terminal end of the α subunit, by strengthening the interaction between the α and β subunits or by mimicking the interaction of β to the α subunit binding site (Binding Site C, Figure 1.2). NS1619 and NS004 are the pioneer drugs and both thought to be BK channel α subunit selective. The BK channel activation property of NS1619 was first observed in aortic smooth muscle cells, where it caused hyperpolarisation of the muscle cells (Olesen *et al.*, 1994), followed shortly by studies showing similar hyperpolarisation in rat ventromedial hypothalamus (Sellers & Ashford, 1994) and rat cortical neurons (Lee *et al.*, 1995).

The substituted benzimidazolone, NS004, is known to cause an increase in mean channel open time, a decrease in interburst interval, and an apparent increase in channel voltage and calcium sensitivity (Biagi *et al.*, 2004a; Biagi *et al.*, 2004b). The effect of both these drugs is increased by levels of cytosolic calcium. By increasing potassium efflux to cause hyperpolarisation of cells, BK channel activators can prevent or reverse cytotoxic cell damage that can result from hyperexcitability hence are of therapeutic interest. In particular to reduce neuronal damage due to traumatic and ischemic events or to neurodegenerative processes as seen in seizure models (Rundfeldt, 1999). Further clinical

applications for these drugs may also be to look at the treatment of urinary incontinence, psychoses and gastroenteric hypermotility (Ghatta *et al.*, 2006) although currently available BK channel activators are used mainly as pharmacological tools rather therapeutic drugs as they lack absolute selectivity for BK channels and are required to be present at high concentrations to elicit their channel activating properties.

BK channel blockers have long been used as experimental tools for examining structural characteristics and mechanism of action. Tetraethylammonium chloride (TEA) is a commonly known BK channel blocking agent that mediates a "flickery" block of BK channels by "sitting" in the pore region and hastens the deactivation process of the channels during repolarization (Li & Aldrich, 2004). It is widely used in research, yet it provides poor selectivity as it also blocks other potassium channel subtypes (Figure 1.2, Binding Site A) (Hermann & Gorman, 1981).

Several toxins have been isolated that block BK channel activity, such as charybdotoxin which also blocks other types of potassium channels including IK channels. Iberiotoxin, a 37 amino acid peptide isolated from the venom of scorpion *Buthus tamulus*, was one of the first examples of a highly selective BK blocker (Candia *et al.*, 1992). Charybdotoxin and iberiotoxin are thought to inhibit potassium current by binding to a site in the external vestibule of the pore thus effectively occluding the extracellular pore (Giangiacomo *et al.*, 1992; Nelson, 1992). Additionally residues within the β1 subunit situated close to the external vestibule of the BK channel α subunit pore are important for creating the high affinity charbydotoxin binding site in these channels (Figure 1.2, Binding Site A) (Hanner *et al.*, 1998).

Tremorgenic mycotoxins are indole alkaloid metabolites produced by fungi belonging to the genera *Penicillium*, *Aspergillus*, and *Claviceps* which elicit intermittent or sustained tremors in vertebrate animals. These natural non-peptide compounds including paxilline, verruculogen and penitrem A demonstrated a selective reversible BK channel blocking profile in radioactive ligand binding and inside-out patch clamp electrophysiology assays in bovine smooth muscle cells (Knaus *et al.*, 1994b). The inhibition of BK channel activity appeared to be by allosteric mechanisms at a site distinct to Site A (Figure 1.2,

Binding Site B), as inhibition of BK channel current was observed when the compounds were applied to the intracellular and extracellular surface of the channel in electrophysiological experiments. This is in direct contrast to the inhibitions observed by charybdotoxin and iberiotoxin, although the block of BK channel activity was more potent when compounds were added at the cytoplasmic face of the channel. The availability of selective BK channel blockers is a fundamental tool for experimental purposes, however it's main therapeutic application appears to be limited to neurological disorders such as depression and memory impairment (Olesen *et al.*, 1994).

1.6 Hypoxia and the role of BK channels

Monitoring of oxygen levels is crucial for cell survival, as it enables living organisms to adapt to the changing physiological environment. There are a number of physiological and pathological conditions under which the supply of oxygen can be limited. For example renal medullary partial pressure of oxygen (pO2) is below 50 mmHg (Johannes *et al.*, 2006) and mean cerebral blood pO2 is closer to 20 mmHg (Hoffman *et al.*, 1999). Pathologically, several cardiorespiratory diseases such as congestive heart failure and chronic

obstructive pulmonary diseases can result in a reduction of systemic or pulmonary pO2.

Chronic deprivation of oxygen (hypoxia) can regulate gene expression of numerous enzymes, growth factors and transporters (Bunn & Poyton, 1996), as well as ion channels (Resnik *et al.*, 2006), which then induce molecular modifications to reduce cellular oxygen dependence and increase oxygen delivery to tissues. These changes in transcriptional activity induced by hypoxia has been shown to rely on oxygen-dependent prolyl and asparaginyl hydroxylases that regulate the activity and nuclear translocation of hypoxia-inducible transcription factors (HIF-1) (Masson & Ratcliffe, 2003). However, protein hydroxylation does not seem to directly participate in acute oxygen sensing, as incubation of PC12 cells (a pheochromocytoma cell line) with dimethyloxalylglycine, a membrane-permeant competitive inhibitor of oxoglutarate that completely inhibits hydroxylases and induces the expression of oxygen-sensitive genes (Del Toro *et al.*, 2003), does not alter the responsiveness of glomus cells to hypoxia (Ortega-Saenz *et al.*, 2003). Heterozygous HIF-$1^{+/-}$ mice, with apparently normal carotid body histology, have impaired responses to low pO2 and adaptability to chronic hypoxia (Kline *et al.*, 2002). In addition the heterozygous HIF knockout mice display blunted changes in pulmonary arterial

myocyte membrane potential and potassium channel density in response to chronic hypoxia (Shimoda *et al.*, 2001). Thus HIFs may be necessary for setting the appropriate level of expression of the oxygen-sensing machinery in carotid body cells and pulmonary myocytes.

In mammals, acute hypoxia triggers rapid respiratory and cardiovascular adjustments to ensure oxygen supply is maintained to critical organs such as the heart and the brain. Although all organs are sensitive to severe hypoxia there are specialized tissues that respond rapidly to acute changes in oxygen tension within the physiologic range. For example, glomus cells of the carotid body and neuroepithelial bodies in the lungs which stimulate rapid ventilation in response to low pO2 (Ortega-Saenz *et al.*, 2007), and smooth muscle cells of the pulmonary arteries that constrict under localised hypoxia thereby optimising ventilation–perfusion matching and gas exchange, whereas systemic arteries dilate, increasing oxygen delivery (Weir *et al.*, 2005). Such changes are dependent on oxygen-sensitive ion channels that arbitrate changes in cellular excitability. These ion channels are thought to be preferentially expressed in excitable cells of specific tissues such as the arterial and airway chemoreceptors (carotid and neuroepithelial bodies), smooth muscle in the pulmonary and systemic

vasculature (Lopez-Barneo *et al.*, 2001; Lopez-Barneo *et al.*, 2004; Resnik *et al.*, 2006), and the brain (Rychkov *et al.*, 1998; Peers & Kemp, 2004).

A hypoxia evoked decrease in potassium channel activity was first described in glomus cells of the carotid body and was membrane delimited (Lopez-Barneo *et al.*, 1988), thus implying that the potassium channel itself was innately sensitive to changes in oxygen tension or that the oxygen sensor was an integral part of the potassium channel complex. There is a general consensus that in glomus cells of the carotid body, neuroepithelial bodies and adrenomedullary chromaffin cells a decrease in oxygen tension inhibits potassium channels (Wyatt *et al.*, 1995; Thompson *et al.*, 1997; Lopez-Barneo *et al.*, 2001) causing membrane depolarisation, leading to activation of voltage-gated calcium channels (Benot & Lopez-Barneo, 1990) thus allowing calcium dependent transmitter release (Urena *et al.*, 1994; Pardal *et al.*, 2000) and inducing the appropriate physiological changes. However, the membrane delimited requirement of the oxygen sensor has been questioned by studies which showed that in fact cytosolic factors are indeed a requirement for hypoxic inhibition of potassium channels (Wyatt & Peers, 1995; Buckler, 1997).

There has been a wide variety of oxygen-sensitive potassium channels identified in various chemoreceptor cells or among cells in between different species (Lopez-Barneo *et al.*, 2001) so it seems unlikely that a single mechanism is responsible for oxygen sensing in all tissues, moreover it is likely that multiple mechanisms are in place to ensure that cellular function is not compromised during hypoxic insult. Potassium channels proposed to be involved include the tandem-pore potassium channels including TASK and THIK (O'Kelly *et al.*, 1999; Buckler *et al.*, 2000; Lewis *et al.*, 2001; Campanucci *et al.*, 2005), the voltage-gated Kv subfamilies (Sanchez *et al.*, 2002; Perez-Garcia *et al.*, 2004) and BK channels (Peers, 1990; Wyatt & Peers, 1995; Williams *et al.*, 2004; McCartney *et al.*, 2005). .

The levels of protein expression of Kv1.5 and Kv3.1 as well as Kv1.1 subunits are reported to be higher in pulmonary resistance arterial myocytes than in other arterial vascular smooth muscle cells (Coppock & Tamkun, 2001). Mice lacking the Kv1.5 subunits have impaired hypoxic pulmonary vassoconstriction and reduced sensitivity of whole cell voltage-gated potassium current in response to hypoxia (Archer *et al.*, 2001), and that antibodies against Kv2.1 diminish oxygen-sensitive currents in rat pulmonary myocytes (Hogg *et al.*, 2002).

Initial studies utilising *in situ* hybridisation, RT-PCR or immunohistochemistry implicate the expression of a number of closely related tandem-pore potassium channels subunits in the carotid body cells including TASK-1, TASK-2, TASK-3, TASK-5, TREK-1, TREK-2 and TRAAK (Buckler *et al.*, 2000; Yamamoto *et al.*, 2002; Kim *et al.*, 2006b; Yamamoto & Taniguchi, 2006). In addition twin-pore potassium channels such as THIK-1 have elicited hypoxia sensitivity in recombinant systems (Fearon *et al.*, 2006). TASK-1 (O'Kelly *et al.*, 1999; Johnson *et al.*, 2004) and TASK-3 (Williams & Buckler, 2004) were later shown to be sensitive to a decrease in oxygen tension whilst TASK-2 (Hartness *et al.*, 2001) and TREK-1 (Buckler & Honore, 2005) were insensitive.

In excised inside-out patches BK channels have been shown to have suppressed activity under conditions of acute hypoxia ($pO2 < 25$ mmHg) , in several different native systems, including the carotid body and alveolar epithelial cells (Peers, 1990; Xie & McCobb, 1998; Pardal *et al.*, 2000; Pardal & Lopez-Barneo, 2002; Jovanovic *et al.*, 2003) and recombinant systems (Lewis *et al.*, 2002; McCartney *et al.*, 2005). Thus implicating BK channels in playing a central role in cellular oxygen sensing and potentially mediating a protective role in some pathological states. For example, during ischemia following stroke or trauma, excessive levels of excitatory neurotransmitters are released (Boris-Moller & Wieloch, 1998; Babot

et al., 2005), leading to excitotoxic neuronal death resulting from excessive calcium influx (Kristian & Siesjo, 1998). Since BK channels can act as an 'emergency brake' to limit increases in intracellular calcium, it has been suggested that they might be part of an endogenous mechanism which enables neuroprotection during ischemic injury. This is evidenced by the fact that in organotypic cerebrocortical and hippocampal slice cultures, blockade of BK channel activity caused increased cell death following oxygen deprivation (Runden-Pran *et al.*, 2002; Katsuki *et al.*, 2005). It is also possible that BK channel mediated neuroprotection is enhanced presynaptically by hyperpolarising the cells to limit excessive excitatory neurotransmitter release. It may be the case that BK channel alternative splice variants such as STREX, which have heightened calcium sensitivity, facilitate stronger neuroprotection during oxygen deprivation.

The concept that BK channel activity is affected by acute and chronic changes in oxygen concentration is widely accepted; however the mechanism of action behind this effect is controversial. Whilst some groups suggest that this is the direct consequence of reduced oxygen tension on the channel and that the mechanism is membrane delimited (Jiang & Haddad, 1994; Riesco-Fagundo *et al.*, 2001; Lewis *et al.*, 2002), others propose that hypoxia indirectly regulates these

channels through changes in second messenger signalling, REDOX changes (Wyatt & Peers, 1995; Wyatt *et al.*, 1995) and production of radical oxygen species (Ward, 2008). Currently there are three key proposed mechanisms which are implicated in the hypoxic inhibition of BK channels: a) Hemoxygenase-2, b) AMP kinase and c) STREX splice variant selectivity.

Hemoxygenase-2 (HO-2) has been implicated to have a role in oxygen sensing and coupling this response to BK channels, as they have co-localised expression (Williams *et al.*, 2004). HO-2 is a membrane bound enzyme that oxidatively breaks down heme to carbon monoxide (CO), biliverdin and iron. This reaction requires oxygen and uses NADPH-cytochrome P450 reductase as the electron donor protein. Coexpression of BK channel α subunits with the β1 subunit in human embryonic kidney (HEK) 293 cells resulted in BK channels that were potently inhibited by hypoxia and that HO-2 gene silencing eliminated this hypoxic inhibition (Williams *et al.*, 2004). The heme binding site on the BK channel α subunit may also interact with the β subunit. It is unclear if this mode of BK channel hypoxic sensitivity requires both the α and β1 subunit, if so expression of the BK channel β1 subunit is largely limited to vascular smooth muscle and are not expressed in cells where native BK channels are reported to be sensitive to hypoxia (Liu *et al.*, 1999). Hence the HO-2 mediated mechanism is

unlikely to be the primary mode of oxygen sensing in physiological systems. Moreover in HO-2 knockout mice the sensitivity of the carotid body to hypoxia is similar to the wildtype response (Ortega-Saenz *et al.*, 2006), although in a separate report the ventilatory response to hypoxia was blunted in HO-2 knockout mice (Adachi *et al.*, 2004).

AMP-activated protein kinase has been proposed as an oxygen-sensing mechanism in carotid body and pulmonary arterioles (Evans et al., 2005; Wyatt et al., 2007) thus connecting cellular function during hypoxia to metabolic status. In this model, hypoxia evokes an increase in AMP/ATP ratio due to the hypoxic inhibition of mitochondrial oxidative-phosphorylation, thus augmenting AMP-activated protein kinase activity via phosphorylation. In turn, AMP kinase activation inhibits BK channels (Wyatt et al., 2007) causing an increase in intracellular calcium concentration, which results in pulmonary arteriolar constriction or carotid body excitation. However these data were obtained in cell-attached patches, thus implying the requirement of an intact cytoplasmic signalling cascade network for hypoxic inhibition of BK channel activity, yet conflicting data on excised-inside out patches (Lewis *et al.*, 2002; Williams *et al.*, 2004) states that cellular constituents near the BK channel are sufficient for

hypoxia mediated inhibition of channel activity or that the channel itself is indeed the oxygen sensor.

The BK channel cysteine-rich STREX splice variant displays clear, reversible inhibition of channel activity during acute hypoxia (pO2 < 25 mmHg) in excised inside-out patches heterologously expressed in HEK293 cells, in contrast to the ZERO and IYF variants that fail to respond to changes in oxygen tension (McCartney *et al.*, 2005). This effect was not manifest in the low oxygen conditions physiologically prevalent in the central nervous system (pO2 ~ 50 mmHg). Therefore revealing that the hypoxia sensitivity of BK channel α subunits is splice-variant-specific. The inhibition of STREX channels was calcium-dependent, as channels exposed to high (10 μM) intracellular free calcium concentration did not respond to hypoxia.

BK channels are also sensitive to redox reagents (Tang *et al.*, 2004; Zhang & Horrigan, 2005) and it was shown that the STREX channel sensitivity of hypoxia was independent of redox regulation as all three variants investigated contained the conserved cysteine residue close to the calcium bowl implicated in redox regulation (Tang *et al.*, 2004), as well as the conserved heme binding motif

(Williams *et al.*, 2004), suggesting that these sites are not required for BK sensitivity to hypoxia. Thus the acute sensitivity of STREX channels is unlikely to involve modulation by conventional REDOX agents or HO-2 but probably a conformational change induced by the decrease in oxygen tension. These investigators implicated a cysteine rich motif (-$C_{23}S_{24}C_{25}$-) within the STREX insert crucial for the hypoxia sensitivity of the STREX channel, as mutation of this motif abolished the response of STREX channels to hypoxia. The STREX insert is cysteine-rich, and so could other cysteine residues within the insert be important hypoxia mediated regulation of STREX channels? Investigating this novel form of regulation would allow placement within a hierarchy of BK hypoxia sensing mechanisms and reveal its potential pathophysiological role.

1.7 Aims of thesis

Alternative pre-mRNA splicing of BK channel α subunits determines the channel phenotype, including its intrinsic voltage dependence and calcium sensitivity thereby providing a mechanism to generate functional diversity of BK channels. The identification and functional characterization of BK channel splice variants has previously relied on the use of low throughput patch clamp electrophysiology. The STREX splice variant enhances BK channel calcium sensitivity and also confers high sensitivity to hypoxia, shown to be dependent upon a cysteine motif (-CSC-) in the STREX insert that is also within a putative phosphorylation motif.

This thesis aims to test the following:

1) To develop a high throughput assay to allow discrimination of different BK channel splice variants and to examine their pharmacology
2) To characterise the role of cysteine residues within the STREX insert that may determine hypoxia sensitivity of the channel
3) To examine the modulation of the hypoxia mediated regulation of STREX channels by intracellular ATP.

Chapter Two: Materials and Methods

2.1 General Reagents

2.1.1 Chemicals

All chemicals were supplied by Sigma-Aldrich Co. Ltd, Poole, Dorset, UK, unless otherwise stated and were of the highest grade available.

2.1.2 Cell culture Reagents

Gibco™ Invitrogen Corporation, Paisley UK

Cell culture media and reagents; Dulbecco's Modified Eagles Medium (DMEM, +L-glutamine), Trypsin EDTA, Geneticin, Hank's Buffered Salt Solution (HBSS) and Foetal Calf Serum.

Lipofectamine 2000.

Harlan Sera-Lab Ltd., Loughborough, Leicestershire, UK

Foetal Calf serum

2.1.3 Molecular Biology Reagents

Abcam Limited, Cambridge UK

Agarose

Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK

Gel electrophoresis power supply

Eppendorf, Cambridge UK

The Perfectprep® Gel Cleanup Kit

Fermentas, York, UK

Restriction enzymes, Pfu DNA polymerase, T4 DNA ligase

Helena Biosciences Europe, Sunderland, Tyne & Wear, UK

HyperladderTm I quantitative DNA ladder

Merck, Darmstadt, Germany

Tryptone

MWG Biotech AG, Ebersberg, Germany

PCR Primers, Sequencing of DNA

New England Biolabs, Herts, UK

Restriction enzymes, Deoxyribonucleotide triphosphates

QIAGEN Ltd. Dorking Surrey, UK

QIAprep® HiSpeed™ Plasmid Maxi Kit, Quicklyse™ Miniprep Kit, Perfectprep™ Gel Elution Kit

Roche, Lewes, East Sussex, UK

Dpn I, Shrimp alkaline phosphatase

2.1.4 FlexStation® II equipment/biochemicals

Molecular Devices, Sunnyvale, California

FLIPR® Membrane Potential Red and Blue dye, FLIPR® Calcium 3, FlexStation® II

Alomone labs, Jerusalem, Israel

rIberiotoxin; 10 μM stock made up in 0.1% Bovine Serum Albumin stored at -20 °C for upto 3 months,

Verruculogen; 1 mM stock made up in Dimethyl solfoxide (DMSO) stored at

-20 °C for upto 3 months,

Penitrem A; 1 mM stock made up in DMSO stored at -20 °C for upto 3 months

Sigma-Aldrich Co. Ltd, Poole, Dorset

Ionomycin; 1 mM stock made up in DMSO stored at -20 °C,

Paxilline; 10 mM stock made up in DMSO stored at -20 °C,

Griener Bio-one Ltd, Stonehouse, Gloucestershire

Poly-D-Lysine coated black 96-well plates

2.1.5 Electrophysiology reagents

Calbiochem, MERCK CHEMICALS LTD, Nottingham UK

BAPTA.(1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) is a calcium-specific chelator

2.2 Cell culture

2.2.1 Cell line

Human Embryonic Kidney (HEK293) cells were used to express cloned variants of the BK channel. HEK293 cells do not endogenously express these channels and retain their protein kinase and phosphatase pathways (Thomas & Smart, 2005), therefore were suitable for my experiments.

2.2.2 Cell culture

HEK293 cells were used over a passage range of 10-40. Cells were passaged every 3 - 5days at approximately 80% confluency and maintained in 25 cm^3 Greiner flasks in a 37 °C incubator with 95% (v/v) air and 5% CO_2. The cells were passaged by removing old media and washing the cells briefly with 5 ml HBSS, then 0.5 ml of Trypsin-EDTA was added to the cells and the flasks were incubated at 37 °C for 1-2 minutes. The cells were then detached gently and resuspended in 1ml of standard HEK293 media (Dulbecco's Modified Eagle's Medium (DMEM) with 10 % foetal calf serum (FCS)) triturating slowly 10-15 times using a 1ml Gilson tip. Once cells were fully resuspended 500 µl of the

suspension was transferred to a new 25 cm^3 tissue culture flask and 4.5ml of HEK293 media was added to give a 1/10 dilution. Antibiotics were used to act as selection factors in the establishment of the stably transfected splice variants or mutants of the BK channel cell line, 0.8 mg/ml Geneticin.

2.2.3 Freezing of HEK293 cells in liquid nitrogen for storage

Cells were passaged as described in 2.2.2. After trypsinisation cells were centrifuged at 5000 G for 1 minute. The cells were then suspended into freezing media comprising of DMEM and 20% FCS plus 5% DMSO. Cells were aliquoted into 1ml cryovials and placed into a cryocontainer at -70 °C overnight before being transferred into liquid nitrogen stores.

2.2.4 Removing of HEK293 cells from liquid nitrogen

The cryovial of cells frozen in liquid nitrogen was thawed rapidly and thoroughly at 37 °C and distributed into a 25 cm^3 flask using 5ml media equilibrated at 37 °C. Media was replaced after ~ 12 hours when the cells had attached to the surface of the flask.

2.2.5 Transient transfections

Lipofectamine 2000 was used to transfect HEK293 cells with the BK channel splice variant or mutant of choice. For electrophysiology experiments HEK293 cells were cultured on glass coverslips (12 mm diameter Chance Glass Ltd, UK) in a sterile 6-well tissue culture plate until approximately 40% confluent. For each transfection reaction, 1.2 μg DNA and 5 μl Lipofectamine 2000 were each diluted separately in 200 μl DMEM without serum, they were then combined, mixed gently, and incubated at room temperature for 20 minutes. The growth media in each well containing the HEK293 cells was removed and discarded. Following the 20 minute incubation, 1ml of HEK293 media was added to the transfection mix which was added to the well. Cells were subsequently incubated for 24 hours at 37 °C in 95% (v/v) air, 5% (v/v) CO_2 before 1 ml of HEK293 media was added to dilute the transfection reaction. They were then used for electrophysiological recordings 3-5 days after transfection.

Cells for FMP assays (Section 3) were similarly cultured and transfected on sterile 6-well tissue culture plates, but without the glass coverslips. Following the 24 hours incubation with the cells at approximately 60% confluency, the transfection mix was removed from the wells, the cells gently washed with 1 ml

HBSS before 0.25 ml of Trypsin-EDTA was added to the wells and the plate incubated at 37 °C for 1-2 minutes. The cells were then detached gently and resuspended in 1 ml of standard HEK293 media. The Biohit electronic multichannel pipettor (50 – 1200 µl) was then used to dispense 100 µl of the cell suspension into each well the Poly-D-Lysine coated black 96-well plate. The plates were incubated for a further 48 hours before being used for Flexstation ® experiments.

2.3 Molecular biology

2.3.1 Standard PCR conditions

Standard polymerase chain reaction (PCR) was carried out in 50 µl reactions, containing 1 U (1 U being the amount of enzyme required to completely digest 1 µg substrate DNA in 60 minutes) of GoTaq DNA polymerase, 10x reaction buffer, 0.2 mM each of dATP, dCTP, dTTP and dGTP, 1.5 mM $MgCl_2$ and 125 ng each of forward and reverse primers. Reactions were generally run for 1 minute at 94 °C, then 25 cycles, using annealing temperatures appropriate to the primers' calculated melting temperature (Tm) and 72 °C extension phases

according to the length of the amplicon, approximately 1 minute per kb, followed by 10 minute at 72 °C after the final cycle.

2.3.2 Site Directed Mutagenesis

Standard PCR reaction was carried out, each reaction contained 2.5 U/μl, 1x reaction buffer, 0.2 mM each of dATP, dCTP, dTTP and dGTP, 10 ng template DNA and 100 ng each of forward and reverse primers, diluted in diethyl pyrocarbonate (DEPC) treated water, which destroys any contaminating DNAase present. Reactions where generally run for 1 minute at 95 °C, then 18 cycles, using annealing temperatures appropriate to the primers' calculated Tm and 72 °C extension phases according to the length of the amplicon, approximately 1 minute per kb, followed by 7 minutes at 68 °C after the final cycle. The PCR product then underwent a Dpn1 (10U/ μl) reaction to eliminate any original unaltered template plasmid, for 1 hour at 37 °C.

2.3.3 Design of primers for site directed mutagenesis of BK constructs

When designing a primer for site directed mutagenesis, a 17-20 base oligonucleotide with the mismatch located in the centre was sufficient for single base mutations. This gives 8-10 perfectly matched nucleotides on either side of the mismatch. Primers had a G-C content of 40-60%, with a higher G-C content at the 5′ prime end than the 3′ prime end. The melting temperature of the primer was calculated from MWG-Biotech (www.mwg-biotech.com/html/s_synthesis/s_overview) and was designed to be closer to 70 °C so as to coincide with the annealing temperatures of the polymerases used in the PCR. The primers designed (Table 2.1) were then ordered from MWG Biotech AG, Ebersberg, Germany.

2.3.4 DNA agarose gel electrophoresis

A 1 % agarose (w/v) gel was prepared in 50 or 100 ml of 1x TBE buffer (45 mM Tris-base, 45 mM Boric acid, 2 mM EDTA, pH 8.0), depending on number of samples. 1 µl of 10,000x SYBR Safe DNA gel stain (Invitrogen Molecular Probes) was added per 10ml of gel, and mixed by swirling, and then the gel

STREX Mutant	Tm (°C)	Sequence 5' - 3'
C12:13A forward	75	-CAA GAG AAT GAG ACG AGC AGC TGC TTT TGA TTG CGG ACG TTC TG-
C12:13A reverse	75	-CAG AAC GTC CGC AAT CAA AAG CAG CTG CTC GTC TCA TTC TCT TG-
C16A forward	71.9	-GCA TGT TGT TTT GAT GCC GGA CGT TCT GAG CGT G-
C16A reverse	71.9	-CAC GCT CAG AAC GTC CGG CAT CAA AAC AAC ATG C-
R21A forward	74.5	-TGC CGA CGT TCT GAG GCT GAC TGC TCG TGC ATG-
R21A reverse	74.5	-CAT GCA CGA GCA GTC AGC CTC AGA ACG TCC GCA ATC-
S24E forward	74.5	-TCT GAG CGT GAC TGC GAG TGC ATG TCA GGC CGT-
S24E reverse	74.5	-ACG GCC TGA CAT GCA CTC GCA GTC ACG CTC AGA AC-
C23A forward	73.6	-TCT GAG CGT GAC GCC TCGTGC ATG TCA GGC-
C23A reverse	73.6	-GCC TGA CAT GCA CGA GGC GTC ACG CTC AGA-
C25A forward	75	-TCT GAG CGT GAC TGC TCG GCC ATG TCA GGC CGT GTG-
C25A reverse	75	-GAC ACG GCC TGA CAT GGC CGA GCA GTC ACG CTC AGA-

Table 2.1 Primers used to amplify regions of murine BK channel STREX variant sequence to enable site-directed mutagenesis and were inserted into pcDNA3 as target vector. For primer sequence of S24A and C23:25A see (McCartney *et al.*, 2005), the C12A , C13A, C16A and C51A were constructed by H. McClafferty.

poured onto a plate to set. DNA samples were mixed with 5 µl loading dye (10x recipe for 100ml: 60% glycerol (v/v), 0.25% bromophenol blue (w/v), 33% 150 mM Tris (pH7.6) (v/v) in H_2O), loaded, and run for 20 - 30 minutes. at 160V using a Bio-Rad model 200/2.0 power supply and Bio-Rad wide min-sub cell GT gel electrophoresis apparatus.

2.3.5 Transformation of chemically competent *E.Coli*

100 µl aliquots of chemically competent *E.Coli* (XL 10) were removed from storage at -80 °C and thawed on ice. 0.5 µl of pcDNA3 plasmid DNA was added and the mixture was incubated on ice for 20min. Subsequently, cells were heat-shocked at 42 °C for 45 seconds and 100 µl of LB medium was added. The mixture was incubated for 30min at 37 °C in a shaking incubator, at 200rpm, then 100 µl of cells were plated onto selective agar containing ampicillin (100µg/µl) and incubated overnight at 37 °C.

2.3.6 Selection of bacterial colonies for production of plasmid DNA

Single colonies grown on selective agar were picked with a sterile pipette tip and resuspended in a 50 µl PCR mixture containing appropriate concentrations

of dNTPs, polymerase buffer, primers and polymerase. Standard protocols were followed for the PCR reaction and positive colonies were analysed further by DNA sequencing. Those that were shown to contain the correct insert were picked using a sterile tip and inoculated into 250 ml LB medium with ampicillin selection (100 μg/μl). This was incubated overnight at 37 °C in a shaking incubator at 200 rpm.

2.3.7 Maxiprep alkaline lysis for plasmid DNA isolation

100 μl of an overnight 5ml culture, or a single colony picked using a sterile pipette tip, was seeded into 100ml LB medium with ampicillin (100μg/μl) for plasmid selection. This was incubated overnight at 37 °C with shaking and subsequently pelleted at 5000 x g for 5 mins at 4 °C. The plasmid DNA was harvested from the pellet using the QIAGEN Hispeed Plasmid Maxi Kit (QIAGEN) according to the manufacturer's instruction.

2.3.8 Miniprep alkaline lysis for plasmid DNA isolation

Individual bacterial colonies were seeded, using a sterile pipette tip, into 5ml of LB medium containing the correct antibiotic for plasmid selection, and then

incubated overnight at 37 °C with 200rpm shaking. 1.5ml of each culture was transferred into a 1.5ml microcentrifuge tube, and centrifuged at 12000 x g for 1min. The LB broth was aspirated and the pellet resuspended by vortexing. Plasmid DNA was harvested using the Qiagen Quicklyse™ Miniprep Kit according to the manufacturer's instructions.

2.3.9 Quantitation of DNA

UV absorbance spectroscopy is a reliable and rapid method for DNA quantification. A 1/100 dilution of the DNA sample in DEPC-treated H_2O was prepared and its' absorbance measured at 260 and 280 nm using a UV spectrophotometer. Purity was assessed using the ratio of A260/A280; an ideal ratio of 1.8 indicated pure dsDNA, whereas lower ratios were indicative of contamination with other nucleic acids or proteins. To calculate the concentration where an OD 260 of 1 indicates DNA at a concentration of 50μg/ml, the following formula was used:

$$[\text{DNA}]\ (\mu g/ml) = OD260 \times 50\ \mu g/ml \times \text{dilution factor}$$

2.3.10 Double restriction digest

20 µl reactions where prepared, each containing 2-10 U of the respective restriction enzyme (New England BioLabs) in the appropriate buffer (New England BioLabs) diluted to 1x with DEPC water, and 0.2-1µg of DNA sample. These reaction mixtures were then incubated at 37 °C for 1hr. DNA gel electrophoresis was used to confirm sizes of digested fragments, which were then, purified using the Eppendorf Perfect Prep gel cleanup kit according to the manufacturer's instructions.

2.3.11 Phosphatase treatment

To prevent re-ligation and to reduce the probability of false positives, phosphate groups were removed from the cut ends of vectors using Shrimp alkaline phosphatase (Roche), 2 µl enzyme and 2 µl buffer to each vector digest. Reaction mixtures were incubated at 37 °C for 10 min and the phosphatase was inactivated via heatshock at 65 °C for 15min.

2.3.12 Ligation of vector and plasmid DNA

Ligation catalyzes the formation of a phosphodiester bond between juxtaposed 5′ phosphate and 3′ hydroxyl termini in duplex DNA. The enzyme will join blunt end and cohesive end termini as well as repair single stranded nicks in duplex DNA. The reaction conditions used were: T4 DNA Ligase 5 U , 1x T4 DNA Ligase Reaction Buffer [50 mM Tris-HCL (pH 7.5), 10 mM MgCl2, 10 mM dithiothreitol, 1 mM ATP, 25ug/ml bovine serum albumin]. The vector:insert DNA ratios were calculated using the equation below to find the optimum ratio for successful ligation. Generally a 1:3 and 1:7 ratio (vector:insert) were used in the equation below;

$$\frac{\text{ng of vector x kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}}$$

= ng of insert

This reaction was carried out at room temperature for 1 hr, before proceeding to transformation of chemically competent *E.coli*.

2.3.13 Preparation of DNA for sequencing

20 µl volumes of cDNA was prepared for absorbance sequencing by MWG-BIOTECH AG, and sent to Ebersberg, Germany. 1-2µg DNA of plasmid, as prepared previously by Maxi/Mini prep alkaline lysis, in 20 µl in 5 mM Tris-HCL, pH 8.0-9.0 as specified by MWG-BIOTECH. (QIAGEN Buffer TE – 10 mM Tris-Cl, pH 8.0; 1 mM EDTA and DEPC water). Primers could be ordered from MWG-BIOTECH for sequencing, or could be enclosed at a concentration of 10pmol/ µl (10 µl). Sequencing results could then be analysed with Vector NTI 10.1.1 (Invitrogen) and Chromas Lite 2.0 (Technelysium Pty Ltd).

2.4 Electrophysiology: Single channel patch clamp

2.4.1 Introduction: Patch clamp

Electrophysiology is the study of transmembrane ion movement and ion channel activity which underlies electrical properties of living cells. A profound advance in the study of ion channels came with the development of the patch clamp methods by Erwin Neher and Bert Sakmann, when they reported the first single channel current records with an acetylcholine-activated channel (Neher & Sakmann, 1976). The real breakthrough came in 1981, when they showed that

clean glass micropipettes filled with the appropriate electrolyte can fuse with a small patch of clean plasma membrane to form a seal of very high resistance and mechanical stability (Hamill *et al.*, 1981), allowing the recording of single channel currents across a living cell membrane. The revolution in the field of ion channel research stimulated by the discovery of this technique was recognised in the award of the Nobel Prize in Physiology or Medicine in 1991 to Erwin Neher and Bert Sakmann "For their discoveries concerning the single ion channels in cells."

Originally the patch clamp technique referred to voltage clamp of a small membrane patch; nowadays it generally refers to both voltage clamp and current clamp. The voltage clamp method allows the measurement of ion flow across a membrane to be recorded as electric current, whilst the membrane voltage is held under experimental control using a feedback amplifier. The key feature of the patch clamp is the formation of the tight seal between the micropipette and the plasma membrane, this seal was named gigaseal as the resistance should typically be in excess of 10^9 Ω. The resistance between pipette and plasma membrane is critical for reducing the electrical background noise so allowing the small electrical currents that are flowing across the membrane to be

resolved. This gigaseal allowed four electrical recording configurations: cell-attached, whole cell recording, outside-out patching and inside-out patching (Figure 2.1). As soon as the pipette is sealed onto the plasma membrane it is possible to record single channels under voltage clamp in cell-attached mode. Due to the great mechanical stability of the seal the patch can be pulled off the cell with the membrane still sealed to the pipette, hence allowing inside-out configuration. Alternatively a cell-attached patch may be ruptured by suction with the pipette still sealed to the cell, this then forms the whole-cell configuration where the recording pipette solution will begin to alter the composition of the cell cytoplasm. Pulling the pipette away from the cell in whole-cell mode results in an outside-out patch. In this thesis I utilised the inside-out patch clamping method for most of my single-channel studies on the BK channel in a recombinant system.

2.4.2 Patch clamp recording

Whole cell and single channel patch clamp recordings of BK channels were made at room temperature (20-24 °C) from isolated HEK293 cells that had been plated on glass coverslips prior to use (see 2.2.5). The glass coverslips were

Figure 2.1
Patch configurations

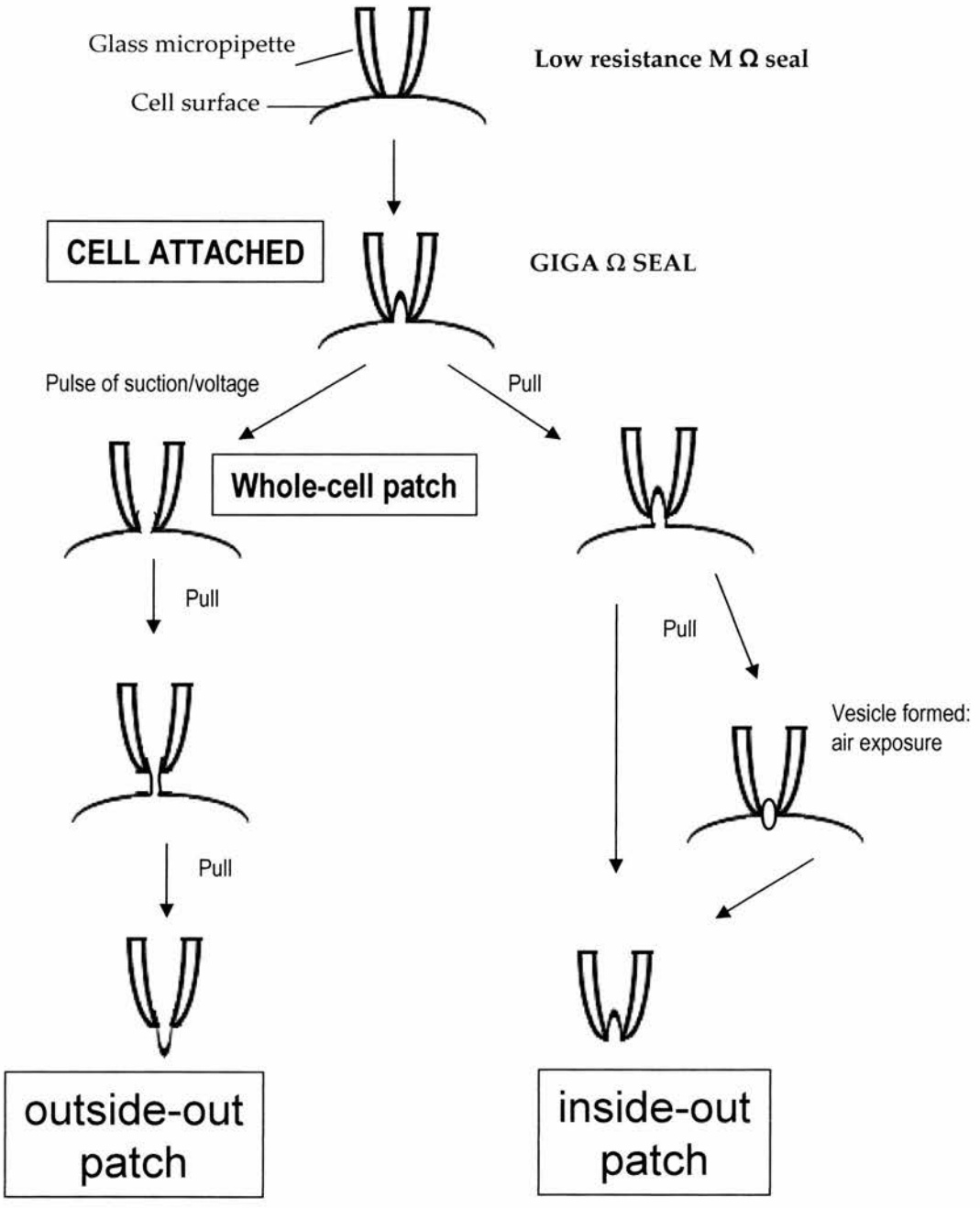


Figure 2.1. Schematic representation of the procedure that leads to different patch configurations in electrophysiology

placed in a perfused recording chamber situated on an (Nikon Diaphot, Japan) inverted microscope. The cells and patch pipettes were observed using x40 phase contrast objective. Mechanical and hydraulic manipulators (Narishige International, Japan, model MHW-3) mounted on the microscope stage provided the control of coarse and fine movement required for patching isolated cells. Channel activity was recorded using an Axopatch-200 patch clamp amplifier (Axon Instruments, USA), an AD converter in the interface board (Digidata 1200, Axon Instruments, USA) and a computer for display and analysis. Borosilicate glass patch pipettes (Harvard Apparatus, Kent) with resistances of 8-12 MΩ for single channel recordings and 4-7 MΩ for whole cell recordings, were mounted on mechanical and hydraulic manipulators. For single channel recordings, recordings were made under voltage clamp with data samples of one minute duration taken every minute at various voltages for the duration of the experiment from patch excision (sampled at 10 kHz and filtered at 5 kHz). All single channel data presented was obtained from excised inside-out membrane patches. Whole cell recordings where made under current clamp, ionomycin (1μM) was bath applied and voltage recorded over 5 minutes (see Section 3).

2.4.3 Solutions and drugs

A range of bath and pipette solutions were used depending on intracellular free calcium $[Ca^{2+}]_i$ required. Inside-out single channel recordings were made in symmetrical potassium concentrations (extracellular and intracellular) with bath solution containing in mM: 5 NaCl, 140 KCl, 2 $MgCl_2$, 30 glucose, 10 HEPES, pH7.3 with free calcium buffered with 1 mM BAPTA. Di-sodium-ATP and 5'-adenylylimidodiphosphate (AMP-PNP) were used at 1 mM, as well as various other cations, all were made up in bath solution. Patches were excised directly into the required solution with ongoing perfusion thus allowing stabilisation of channel activity. Whole cell recordings were in asymmetrical solutions, the pipette solution contained (in mM): 140 KCl; 5 NaCl; 1 $MgCl_2$; 10 Glucose;10 HEPES; 1 BAPTA; and $CaCl_2$ to give a free calcium concentration of 1μM (pH 7.3) The bath solution contained (in mM) : 135 NaCl; 5 KCl; 2 $CaCl_2$; 1 $MgCl_2$; 10 Glucose; 10 HEPES.

2.4.4 Oxygen tension measurement

The changes in oxygen tension of the bath during perfusion with hypoxic saline were recorded using an oxygen microelectrode which had a response time of

less than 10 seconds (World Precision Instruments, Model IS02). The oxygen electrode was calibrated on normoxic saline containing in mM: 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 20 glucose, 10 HEPES at pH7.3 Po_2 = 150 mmHg/100% and saline made anoxic by bubbling with nitrogen gas giving Po_2 = 0 mmHg/0%.

2.4.5 Optimisation of hypoxia protocol

Inside-out patch clamp recordings were used to investigate the effects of hypoxia on BK channel activity using continuous prolonged perfusion of hypoxic bath solution. Hypoxic solutions were obtained by bubbling bath solution with nitrogen gas for at least 10 minutes prior to application to bath, the resulting solution being in the hypoxic range with a Po_2 between 15 and 25 mmHg. Excised inside-out patches were held at the required voltage for 10 minutes to allow stabilisation of ion channel activity prior to perfusion with the hypoxic solution at a rate of 2 ml/min hence allowing complete exchange of bath volume within a minute. The hypoxic bath solution was then washed out using normoxic solution for 10 minutes at the same perfusion rate.

It was critical to maintain the hypoxic oxygen tension in the bath during experiments and it was found that different baths and indeed different tubing connecting the bath to the perfusion system significantly altered the oxygen tension. The tubing that connected the perfusion system with the bath was first investigated. Different tubing was connected to bath A (Figure 2.2) and perfused with hypoxic saline oxygen tension was then measured with the ISO2 oxygen meter (World Precision Instruments Inc.) placed at the point indicated (Table 2.2). It was found that the gas permeability of the tubing material as well as the inner and outer diameter of the tubing made a significant difference to the oxygen tension of the bath (Table 2.2) ideal tension required was < 3% [O_2], which corresponds to ~20 mmHg. Hence it was decided that the Altec Santoprene tubing (inner diameter 0.8 mm and wall 1.6 mm) would be used for all hypoxia experiments as it gave a 3% oxygen concentration, as the Pharmed ® tubing which gave 2.6% oxygen tension was no longer commercially available.

Three differently shaped baths were then investigated using the Altec Santoprene tubing; all three surprisingly gave very different oxygen tension readings when measured in a similar fashion. Oxygen tension maps using hypoxic saline and the ISO2 oxygen meter were produced. Bath A had an inlet

Figure 2.2
Bath Oxygen tension: Bath A

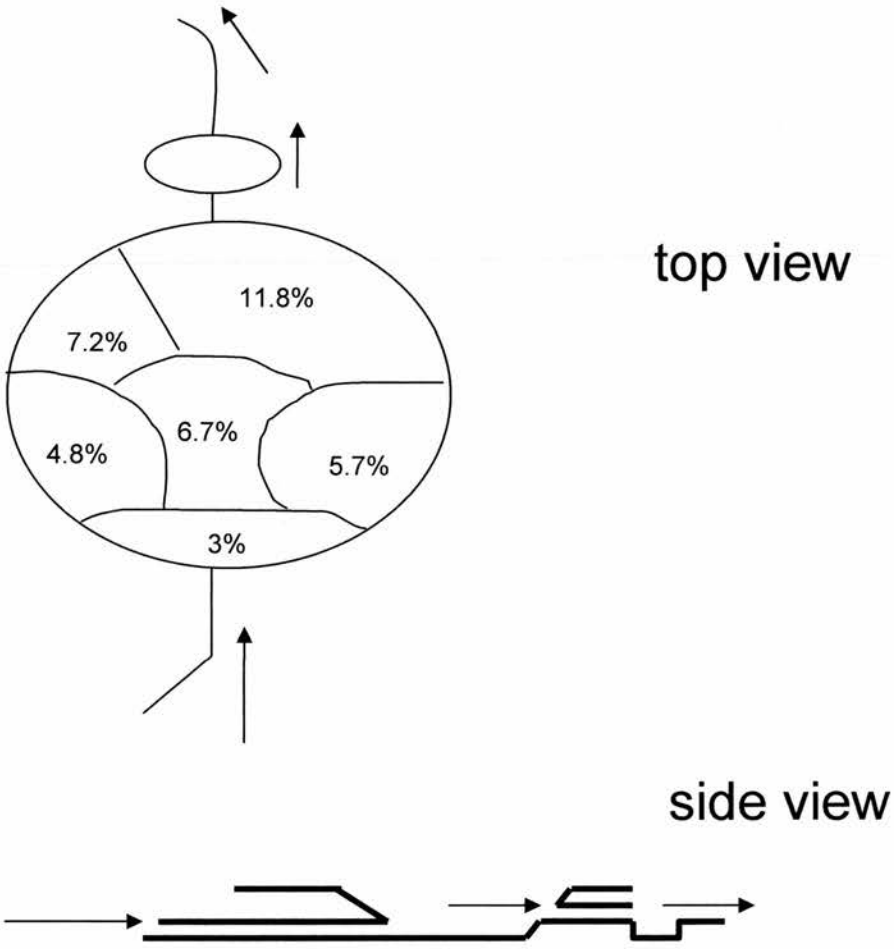


Figure 2.2 Measurement of the percentage of oxygen tension using bath A. The top view illustrates the shape of the bath, with the side veiw depicting the position of the inlet, outlet and approximate bath depths. Arrows indicate the direction of the flow of perfused solution (not to scale).

Table 2.2
Oxygen tension of various tubing

Tubing	% O_2
Tygon	6.0%
ID 1/16", OD 1/8, Wall 1/32	
Pharmed	5.2%
ID 1/8", OD 3/16, Wall 1/32	
Altec - Santoprene	4.9%
ID 1.8mm, Wall 3.2mm	
Pharmed	3.9%
ID 1/8", OD 1/4, Wall 1/16	
Altec - Santoprene	3.0%
ID 0.8mm, Wall 1.6mm	
Pharmed	2.6%
ID 3.25mm, Wall 1.6mm	

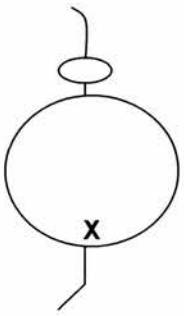


Table 2.2 Measurement of the oxygen tension of various tubing using bath A. Red circle highlights the tubing used for the final hypoxia protocol

that perfused directly into the bottom of the bath (Figure 2.2) with the walls graduated around the circular bath, and the outlet was elevated thus allowing connection with the external suction pump. It provided the correct oxygen concentration required but only towards the inlet, due to the graduated shape of the bath oxygen from the air could be dissolving into the bath solution that could increase the oxygen tension.

Bath B had an elevated inlet and outlet with a well-like shape of the circular bath (Figure 2.3), this bath failed to show any areas that had the ideal oxygen concentration. This could be due to the height at which hypoxic solution came into the bath, dissolving of oxygen from the air was enhanced. In addition, the suction pump was connected to an outlet that was also elevated meaning that the hypoxic solution remained in the bath longer hence lowering the perfusion rate. Bath C (Figure 2.4) allowed inflowing solution in through a tube that opened up at the bottom of the circular bath, the outlet was unique in that it was V-shaped and allowed the solution to drawn directly along the bottom of the bath and pumped out. This unique shape of the bath gave minimal chance of the hypoxic solution perfusing with oxygen in the air and had <3% oxygen tension throughout all areas of the bath. It was therefore concluded to use bath

Figure 2.3
Bath Oxygen tension: Bath B

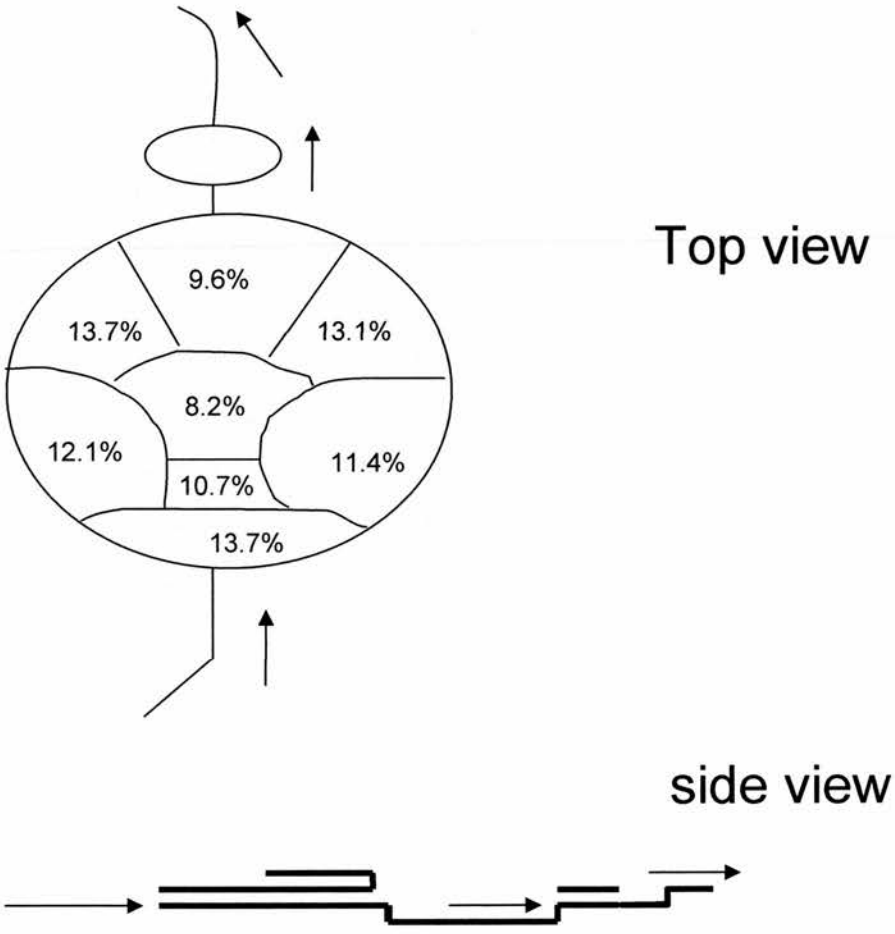


Figure 2.3 Measurement of the percentage of oxygen tension using bath B. The top view illustrates the shape of the bath, with the side veiw depicting the position of the inlet, outlet and approximate bath depths. Arrows indicate the direction of the flow of perfused solution (not to scale).

Figure 2.4
Bath Oxygen tension: Bath C

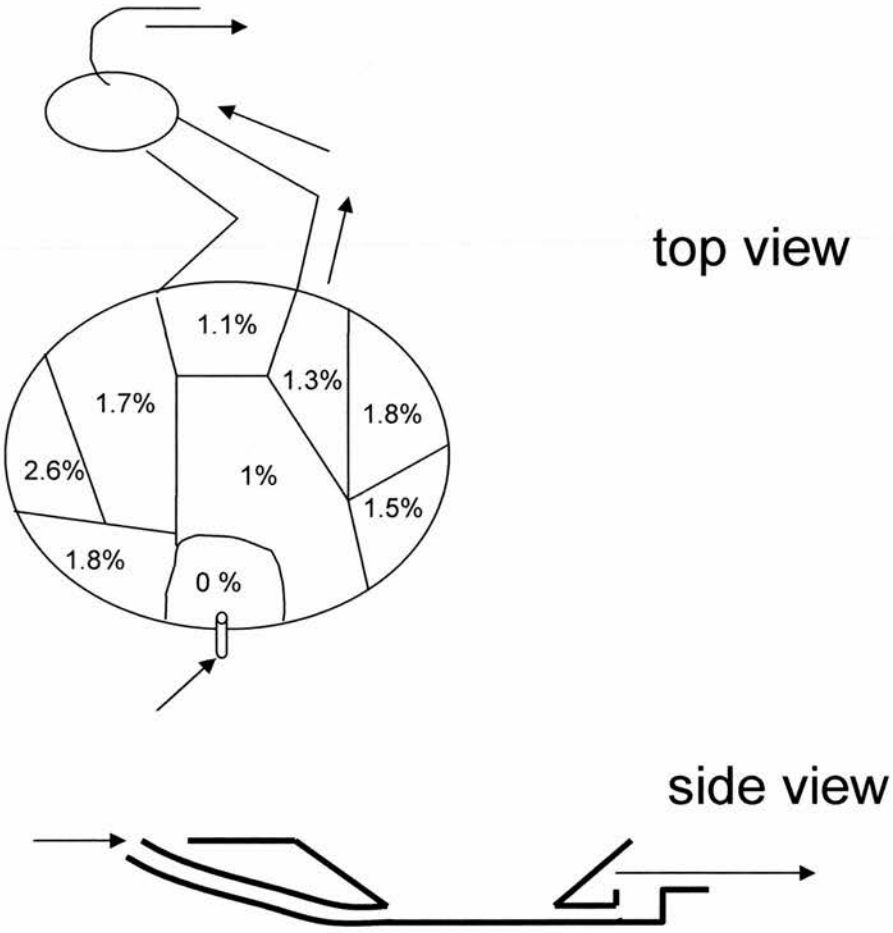


Figure 2.4 Measurement of the percentage of oxygen tension using bath C. The top view illustrates the shape of the bath, with the side veiw depicting the position of the inlet, outlet and approximate depths of the bath. Arrows indicate the direction of the flow of perfused solution (not to scale).

C for hypoxia experiments as it provided the optimum and most static oxygen tension over a period of 30 minutes; the duration of the hypoxia experiments.

Final Hypoxia protocol thus used bath C and Altec Santoprene tubing (inner diameter 0.8 mm and wall thickness 1.6 mm) with the hypoxic solution bubbled with nitrogen gas for > 10 minutes prior to perfusion at 2 ml/min. Patches excised were exposed to normoxic bath solution for 10 minutes to allow stabilisation of channel activity, then hypoxic solution was perfused for 10 minute followed by 10 minutes washout period with normoxic solution.

2.5 Data analysis

Patch clamp data was recorded using pClamp 9 and analysed using Win EDR version 2.3.9 (Dempster, J., University of Strathclyde, UK). FlexStation® II data was recorded using SoftMax Pro version 4.8 (© Molecular Devices 2004) and analysed using Igor version 4.0.6.1 (© Wavemetrics Inc 1988-2002 GraphPad Prism version 4.00 was used for all statistical analysis, with ANOVA and t tests, and for producing graphs. Single channel open probability (Po) was calculated using the following equation: Nf x Po = I/i. Where Nf = number of channels present in patch, I = total current and i = single channel amplitude.

Chapter Three: Development of FMP assay for BK channel splice variants

3.1 Introduction

BK channels are expressed in many different types of excitable cells and have significant roles in the regulation of frequency of firing, action potential repolarization, and after hyperpolarization (Kaczorowski *et al.*, 1996; Vergara *et al.*, 1998). Thus they are involved in a variety of physiological functions such as regulating vascular smooth muscle tone (Brayden & Nelson, 1992; Liu *et al.*, 1997), immunity (Ahluwalia *et al.*, 2004), neurotransmission (Robitaille *et al.*, 1993) and uresis (Burdyga & Wray, 2005). A deficiency of the α subunit (KCNMA1) in mice can cause not only diseases related to smooth muscle dysfunctions, such as elevated blood pressure, overactive bladder, urinary incontinence, and erectile dysfunction, but also cerebellar ataxia, hearing loss, and hyperaldosteronism (Meredith *et al.*, 2004; Sausbier *et al.*, 2004; Sausbier *et al.*, 2005).

Studies using animal models have allowed a recent explosion of data implicating the functional role of BK channels in diseases, in rat models BK channel openers have been shown to protect the brain against stroke (Gribkoff *et*

al., 2001b). BK channel β1 subunit expression is decreased in a hypertension model (Amberg & Santana, 2003) whilst in ageing arteries BK channel α and β1 subunits are both downregulated (Marijic *et al.*, 2001; Nishimaru *et al.*, 2004), explaining the increase in coronary reactivity in the elderly. A recent study elucidated that a gain of function mutation in the RCK1 domain of the BK channel α subunit has been implicated in paroxysmal dyskinesia (Du et al., 2005). BK channels are therefore attractive targets for drug research and development for the treatment of a variety of smooth muscle and brain diseases.

Several sites of alternative pre-mRNA splicing within the BK channel α subunit have been described, the majority of which are located within the intracellular C-terminal domain of the channel (see Figure 1.4) (Shipston, 2001). Analysis of these alternatively spliced variants has revealed the dramatic modifications they can cause in the functional properties of BK channels, including changes in calcium and voltage sensitivity (Liu *et al.*, 2002), regulation by protein phosphorylation (Tian *et al.*, 2001a) and other intracellular signalling cascades (Tian *et al.*, 2001a; Erxleben *et al.*, 2002a) as well as cell surface expression. A recent study focusing on alternatively spliced variants of the c2 splice site of BK has highlighted different intrinsic voltage and calcium sensing properties

between four different variants; the cysteine rich 59 amino acid STREX (e21), 29 amino acid insert e22, a three-amino acid (IYF) insert e20 and insertless null variant known as ZERO (Chen *et al.*, 2005). The STREX variant was expressed at the highest levels in mouse adult endocrine tissues and e22 more so during embryonic stages of mouse development. The variants were differentially sensitive to phosphorylation by endogenous cAMP-dependent protein kinase; ZERO, e20, and e22 variants all underwent activation whereas the STREX variant is inhibited. These data demonstrate how functional diversity can be produced from a channel that is encoded by a single gene.

Over recent years the discovery of new BK channel subunits and variants has encouraged the development of new assays to characterise their physiological and pharmacological properties. For example cell viability assays in yeast and mammalian cell lines, which directly relate cell survival to flux of potassium ions and ion channel activity (Anderson *et al.*, 1994), have been valuable in identifying potassium channel blockers in high-throughput screens. Radioligand binding studies have also revealed distinct binding sites for different classes of channel modulators (Catterall, 1995). Radiotracers have long been used to follow ion flux through channels in cells, in glioma cells IK channel

(intermediate-conductance calcium activated potassium channel) activity through ^{86}Rb influx, following addition of the calcium ionophore ionomycin, has proved as a useful technique for screening toxins and crude venoms (de-Allie *et al.*, 1996).

Charybdotoxin although not a selective blocker of BK channels is known to bind to a site in the external vestibule of the BK channel pore thus occluding the extracellular pore, similar to the highly-specific BK channel blocker iberiotoxin (Giangiacomo *et al.*, 1992). Disruption of charybdotoxin binding in radioligand binding assays has been used in the past to identify specific BK channel blockers (Knaus *et al.*, 1994b). BK channel activity has also been investigated in lipid bilayers (Candia *et al.*, 1992) and patch clamp electrophysiology (Miller *et al.*, 1985; Galvez *et al.*, 1990; Knaus *et al.*, 1994a).

To identify specific pharmacologically active molecules that are able to selectively modify channels for therapeutic use, it is necessary to develop functional assays that are compatible for high-throughput screening (HTS) of large compound libraries. Although patch and whole cell clamp recording are

the most sensitive methods to characterize the gating, ion permeability and drug interactions and is thus used in the late stages of the pre-clinical and development process, it is not compatible for high-throughput assays.

High-throughput screening for ion-channel function requires sensitive, simple assays and instrumentation that will report ion channel activity in living cells; one such method is using voltage-sensitive dyes. There are many such dyes commercially available such as the pyridium dye 1-(3-sulfonatopropyl)-4-[β[2-(di-n-octylamino)-6-naphthyl]vinyl]pyridinium betaine (Di-8-ANEPPS) (Fluhler *et al.*, 1985; Zhang *et al.*, 1998) and the bisoxonol dye, bis-(1,3-dibutylbarbituric acid)trimethine ($DiBAC_4(3)$) (Apell & Bersch, 1987; Shapiro, 2000). However, these traditional dyes have several limitations, including slow dye loading and response times (Baxter *et al.*, 2002), temperature sensitivity (Gonzalez *et al.*, 1999) and interactions with cytosolic proteins (Epps *et al.*, 1994). In addition micro-molar concentrations of Di-8-ANEPPS have recently been shown to increase the open probability of BK channels in pituitary tumour cells, thereby enhancing the activity of the channel (Wu *et al.*, 2008). Di-8-ANEPPS is a lipid analogue that is preferentially retained in the outer leaflet of cell membranes. At high concentrations it can affect membrane dipole potential (Clarke & Kane, 1997)

thus affecting lateral stress associated with the lipid bilayer. As no specific binding site has been identified on BK channels for the dye it is possible that the dye mediates an effect on ion channels by modulating the behaviour of the membrane that surrounds the channel protein rather than binding to the channel protein per se. Also, the slow response oxonol dye $DiBAC_4(3)$ at 300 nM was recently reported to activate BK channels associated with the β1 and β4 but not the BK α subunit alone (Morimoto et al., 2007). This suggests that this voltage sensor dye should be used at low concentrations to avoid artefacts caused by dye-protein interactions.

The FLIPR (fluorescent-imaging plate reader) Membrane Potential (FMP) dyes (Molecular Devices, Sunnyvale CA) are propriety voltage-sensitive bis-oxonol dyes used to assess relative changes in membrane potential. They deliver higher throughput while showing good correlation with manual patch clamping data (Baxter *et al.*, 2002). The FMP dye has a stronger signal-to-noise ratio, is less temperature sensitive and the dye response itself takes seconds. In addition it has demonstrated lower incidence of interference from quenching and artefacts in comparison to $DiBAC_4(3)$, in potassium channel assays (Whiteaker *et al.*,

2001). Therefore it was utilised as the voltage-sensitive dye of choice for investigating the properties of BK channels in this study.

The dye enters depolarized cells where it binds to intracellular proteins or membranes and exhibits enhanced fluorescence (Figure 3.1). Depolarization results in increased influx of the anionic dye and thus an increase in fluorescence. Conversely, hyperpolarisation is indicated by a decrease in fluorescence. Hence the effect of opening and closing ion channels on membrane potential can thus be followed. Extracellular dye can significantly contribute to background fluorescence, so to overcome this, cell impermeable dye quenchers are included in the assay buffer to minimise the excitation of any extracellular fluorescent dye.

Figure 3.1
Fluorescent response of the FMP voltage-sensitive dye

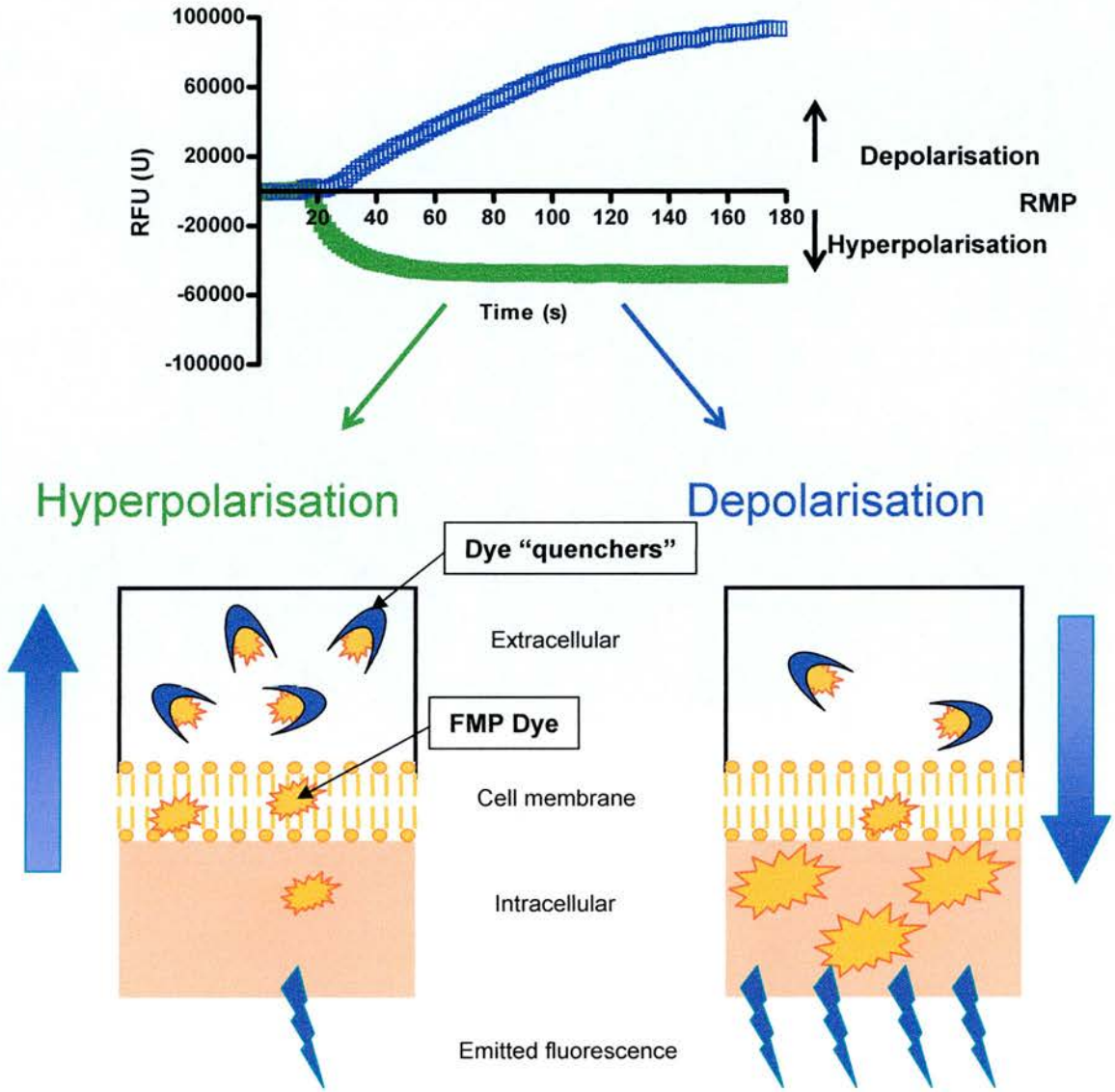


Figure 3.1 FLIPR (fluorescent-imaging plate reader) Membrane Potential (FMP) dye emits fluorescence in response to changes in membrane potential of the cell. Depolarisation of the cell membrane results in compartmentalization of the dye intracellularly which enhances fluorescence. Upon hyperpolarisation the dye remains at the extracellular face of the cell membrane where it is quenched by quenchers present in the dye buffer therefore reducing fluorescence.

Alternative pre-mRNA splicing of the BK α subunits determines the channel phenotype, including its intrinsic voltage dependence and calcium sensitivity (Chen *et al.*, 2005). The STREX (STRess regulated EXon) splice variant of BK contains a 59 amino acid insert at site 2 (Figure 1.4), which results in increased calcium sensitivity in comparison to the ZERO and e22 variant. The identification and characterization of BK channel splice variants depends on the use of low throughput patch clamp electrophysiology. Therefore I aimed to develop a robust, high throughput assay to allow rapid screening of BK splice variants and to test their pharmacological properties.

3.2 Results

3.2.1 FMP methods

HEK293 cells were transiently transfected with the BK channel splice variant of choice using the lipofectamine method (see Section 2.2.5). They were then seeded into black clear bottom Poly-D-lysine coated 96-well plates (Greiner Bio-One Ltd, Gloucestershire UK) 48 hours prior to experiments to allow settling and attachment of cells. The dye was prepared as recommended by the manufacturers with 2 mM extracellular free calcium. When the cells had

reached 90 – 100 % confluency the culture medium was removed by aspiration and cells were incubated for 30 minutes at 37 °C to allow dye loading into the cell membrane. Ionomycin is a known calcium ionophore (Haug & Sand, 1997) and was used as the stimulus of choice to activate BK channels, at 1 μM (0.01% DMSO as vehicle) to stimulate channel activity via influx of calcium ions. The automated liquid-handling function of the FlexStation® II was programmed to run the FMP protocol; to administer 50 μl of ionomycin to a total well volume of 200 μl, 16 seconds after the experiment began and to read fluorescence changes for 3 minutes.

3.2.2 Optimisation and development of FMP assay

Here, I report for the first time that a voltage-sensitive assay has been developed for BK channels (Yamada *et al.*, 2001; Morimoto *et al.*, 2007) exploiting a fluorescent membrane potential (FMP) dye (Molecular Devices, Sunnyvale CA) , which required extensive optimisation and development. The FMP assay was initially optimized on a 96-well plate format using the FMP dye, on the automated fluid-handling fluorometer FlexStation® II (Figure 3.2, Molecular Devices, Sunnyvale CA).

Figure 3.2
FlexStation® II

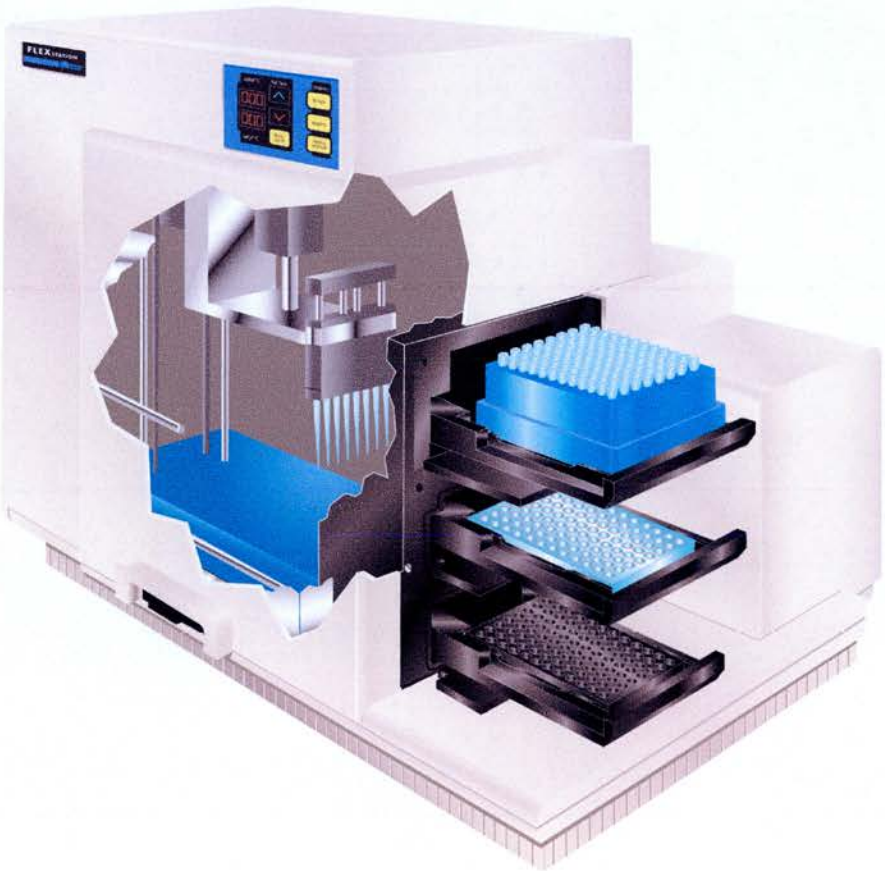


Figure 3.2 FlexStation® II is a benchtop scanning fluorometer that provides both top- or bottom-read functions. It's integrated 8-channel pipettor, is able to transfer compounds from one microplate to the assay plate, allows prompt kinetic assays such as calcium flux and membrane potential. The integrated fluidics platform allows precise compound transfer using consumable pipette tips to completely avoid cross-contamination.

(Figure adapted from Molecular Devices.)

The resulting increase in STREX channel activity caused a hyperpolarisation in transfected cells which was detected by the FMP dye as a decrease in fluorescence; non-transfected HEK293 cells elicited a depolarisation in response to the influx of calcium ions and increased fluorescence (Figure 3.3). Control experiments on the FMP assay were conducted to investigate if there was an effect of the solvent DMSO on STREX expressing HEK293 cells, the same concentration of DMSO (0.01 %) lacking the ionomycin drug was administered and did not show any significant changes in baseline fluorescence (Figure 3.3).

A primary concern in HTS assays used to investigate pharmacological properties is the reliability of the data, thus the stability and well-to-well variation must be optimal. The experimental Z′ parameter (Zhang *et al.*, 1999), which is a statistical parameter widely used to characterize the robustness of an assay and which consequently allows setting of an upper and lower limit to the assay window, which usually correspond to the negative and positive controls respectively.

$$Z' = 1 - \frac{3 \times (\text{standard deviation upper} - \text{standard deviation lower})}{3 \times (\text{mean upper} - \text{mean lower})}$$

Z′ values of 0.3 – 1 are acceptable standards of assay reliability. Z′ analysis was carried out on all optimization plates and gave satisfactory values of > 0.3 to pass.

Calcium influx was measured using the Calcium 3 dye (Molecular Devices, Sunnyvale CA) to verify that the resulting hyperpolarisation and depolarisation was indeed due to the influx of calcium ions. The assay was run according to manufacturer's protocol, as with the FMP assay the dye required 30 minutes loading into the cell membrane. Administration of ionomycin elicited an increase in fluorescence which represented an increase in intracellular calcium concentration. There were no significant differences in calcium influx between the transfected and non-transfected HEK293 cells (Figure 3.4) verifying that lipofectamine transfection of HEK293 cells with BK channels does not alter the ionomycin-induced calcium entry into the cell.

The ionomycin-induced hyperpolarisation response in the FMP assay was then investigated in HEK293 cells transfected with STREX in the absence of extracellular calcium (by buffering with the calcium chelator BAPTA) and at a variety of extracellular calcium concentrations 2 mM (control), 3 mM and 5mM

to observe the effects of extracellular calcium availability. The data obtained was analyzed at the 70 second time point, as this was considered the time point at which the maximal ionomycin-induced hyperpolarisation was observed in the FMP assay. All data analyses hereafter were accordingly compared and analysed at this timepoint. Ionomycin-induced responses were expressed as a percentage of the control (2 mM extracellular) calcium concentration, 3 and 5 mM free extracellular calcium concentrations induced similar responses to control calcium concentration (Figure 3.5). In the calcium free FMP assay a small transient hyperpolarisation was observed in response to ionomycin addition that was significantly different when compared to all calcium concentrations ($p<0.001$ ANOVA with Tukey posthoc). This could potentially be explained by the predisposition of ionomycin to behave as weaker ionophore for other cations (Kauffman *et al.*, 1980; Erdahl *et al.*, 2000), however the degree of the hyperpolarisation was not as seen in the control extracellular calcium concentration (2 mM) verifying that the calcium influx mediated by ionomycin into HEK293 cells expressing STREX causes a robust activation of the channels and thereby hyperpolarisation.

Figure 3.3
Ionomycin stimulates membrane hyperpolarisation in HEK293 cells expressing BK channel STREX variant

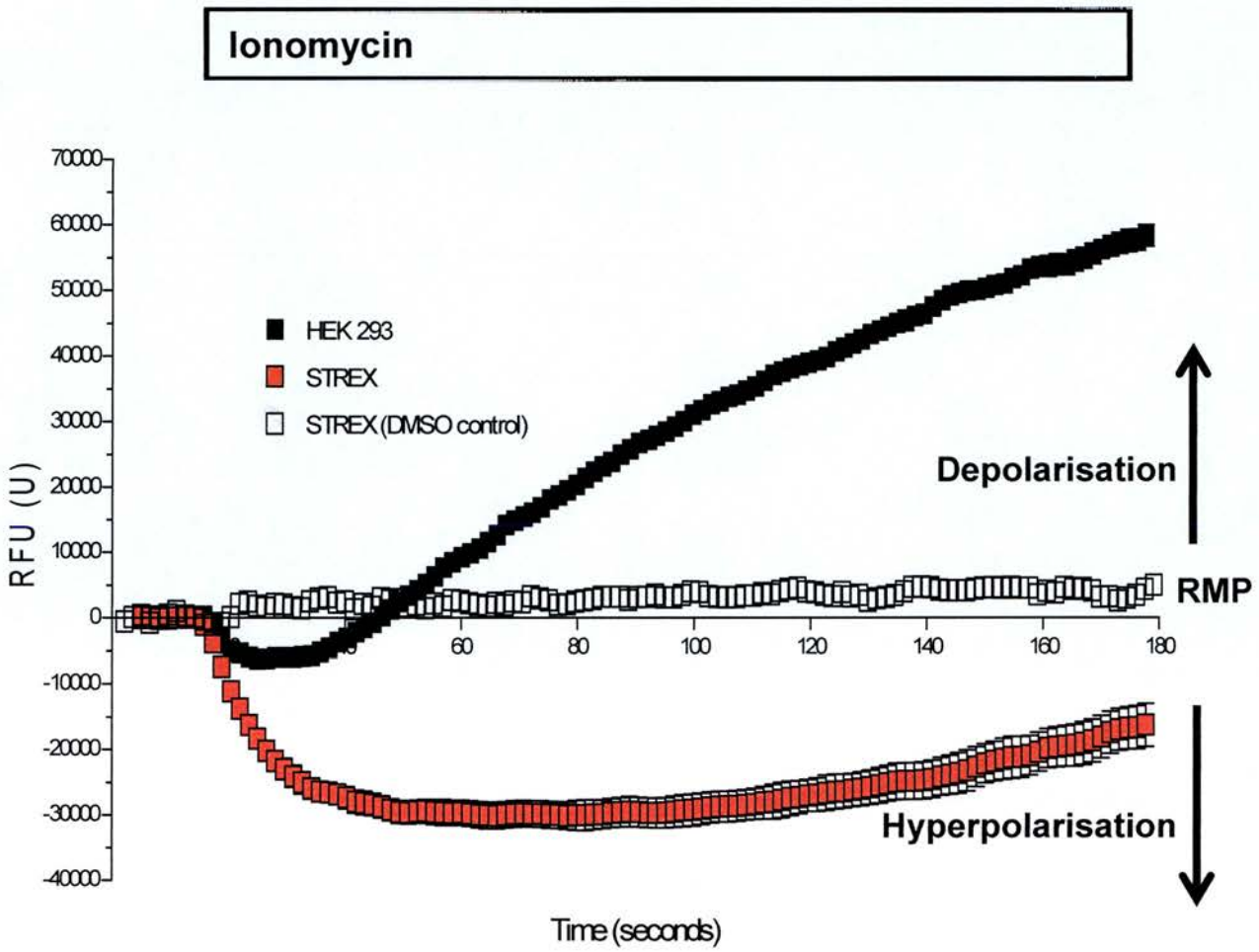


Figure 3.3 Ionomycin (1μM) was automatically administered at t = 16s, which induced a significant prolonged hyperpolarisation in HEK293 cells transfected with the STREX variant but not in untransfected controls. This response was not due to any solvent effect as saline & DMSO control did not exhibit any change in fluorescence. Data are mean ± s.e.m n=8. (RMP = resting membrane potential)

Figure 3.4
Ionomycin stimulates an increase in intracellular free calcium in HEK293 cells

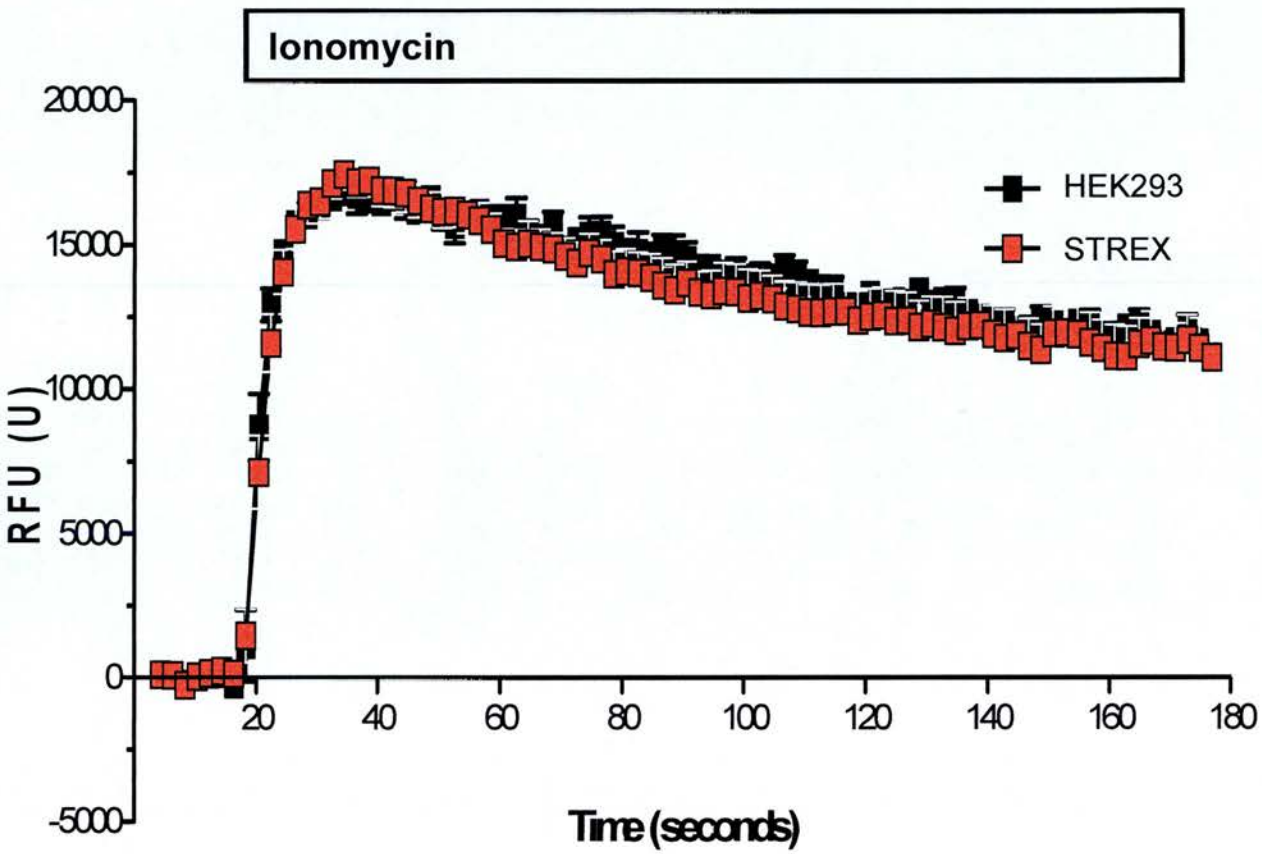


Figure 3.4 Ionomycin administration resulted in an increase in intracellular free calcium in HEK293 cells as measured using Calcium 3 dye (Molecular Probes), shown here by an increase in fluorescence. Expression of the STREX BK channel splice variant had no significant effect on the ionomycin-induced calcium influx in HEK293 cells. Data are mean ± s.e.m n=3.

Figure 3.5
Ionomycin-induced hyperpolarisation of STREX channels in various extracellular calcium concentrations

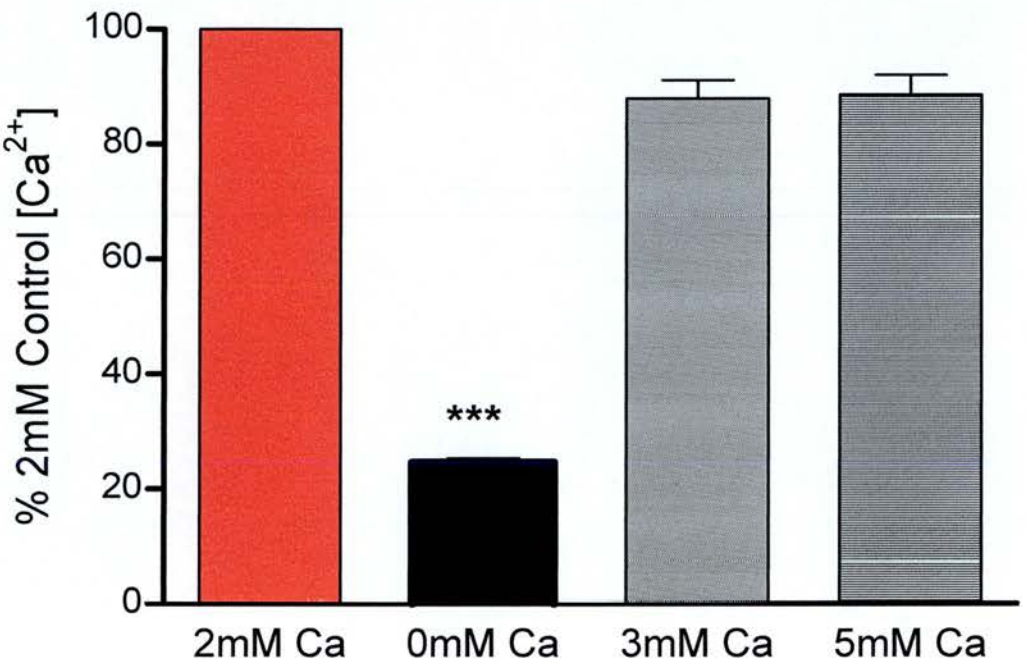


Figure 3.5 Ionomycin-induced similar hyperpolarisation in 2, 3 and 5 mM extracellular calcium concentrations in HEK293 cells expressing STREX channels. In 0 mM buffered extracellular calcium a small transient hyperpolarisation was observed that was significantly different when compared to all calcium concentrations *** $p<0.001$ ANOVA with Tukey posthoc. Data are mean ± s.e.m n=8.

There are two different FMP dyes available, red and blue, as dye interaction can be a potential issue when screening large libraries of compounds. Both dyes were tested to see which would provide the more robust reproducible response. A batch of HEK293 cells transiently transfected with STREX were seeded onto a 96-well plate, half the plate was loaded with the red FMP dye and half with blue therefore maintaining identical treatment of cells bar dye loading. Both dyes exhibited a similar extent of hyperpolarisation at the 70 second timepoint in response to ionomycin administration (Figure 3.6a) thus showing that either could be used in the FMP assay. The FlexStation® II can be set to run experiments at different temperatures so the FMP assay was run at 22 °C (i.e. room temperature) and 37 °C to compare any differences in response. At 37 °C the degree of hyperpolarisation of STREX to ionomycin was in fact reduced in comparison to the response at 22 °C (at t = 70 seconds, Figure 3.6b $p<0.001$ Students paired t-test). This could be because at the higher temperature the calcium ion homeostasis may be affected i.e. the higher temperature somehow affected the calcium ion influx and therefore the degree of STREX stimulation was significantly less, or that the dye interaction with intracellular proteins was altered. This highlighted the somewhat temperature sensitivity of the FMP

assay and FMP assay protocol was therefore set at 22 °C to correlate with electrophysiological analysis.

The ionomycin-induced hyperpolarisation was investigated using current clamp whole cell electrophysiology in transiently transfected HEK293 cells, to validate the use of ionomycin as an appropriate stimulus to activate BK channels and to confirm the membrane hyperpolarisation. All experiments were carried out at room temperature (22 °C) in accordance with the FMP protocol. Control recordings made prior to ionomycin application, determined that the resting membrane potential was not significantly different between the transfected and non-transfected cells (HEK293 -30 ± 4 mV, ZERO -29 ± 2 mV and STREX -34 ± 6 mV). Upon administration, ionomycin elicited a greater hyperpolarisation in STREX expressing cells than in those expressing the ZERO BK variant. Values were compared at the optimal response after exposure to ionomycin at 70 seconds, as with the FMP assay (-82 ± 3 mV and -48 ± 5 mV respectively *** $p<0.001$ ANOVA with Tukey posthoc test). Control experiments on non-transfected HEK293 showed a depolarisation upon ionomycin addition (Figure 3.7).

This further verified that the decrease in fluorescence seen in STREX transfected HEK293 cells in the FMP assay is indeed attributable to ionomycin-induced channel activation which leads to hyperpolarisation of the cell membrane. Assuming a linear response, the ionomycin induced hyperpolarisation in membrane potential in HEK293 cells expressing STREX channels was 51 ± 3 mV. This was correlated with a mean 35 ± 3 % change in dye emission (% change in Relative Fluorescent Units (RFU)) in the FMP assay suggesting that the sensitivity of the assay is approximately a 1.5 mV change in membrane potential for a 1% change in RFU. In addition, using the same concentration of dye as in the FMP assay, we confirmed that the bis-oxonol dye itself does not activate the channel in patch clamp assays (n=2), as reported for other voltage sensor dyes (Morimoto *et al.*, 2007).

Figure 3.6
Comparison of different FMP dyes and temperature

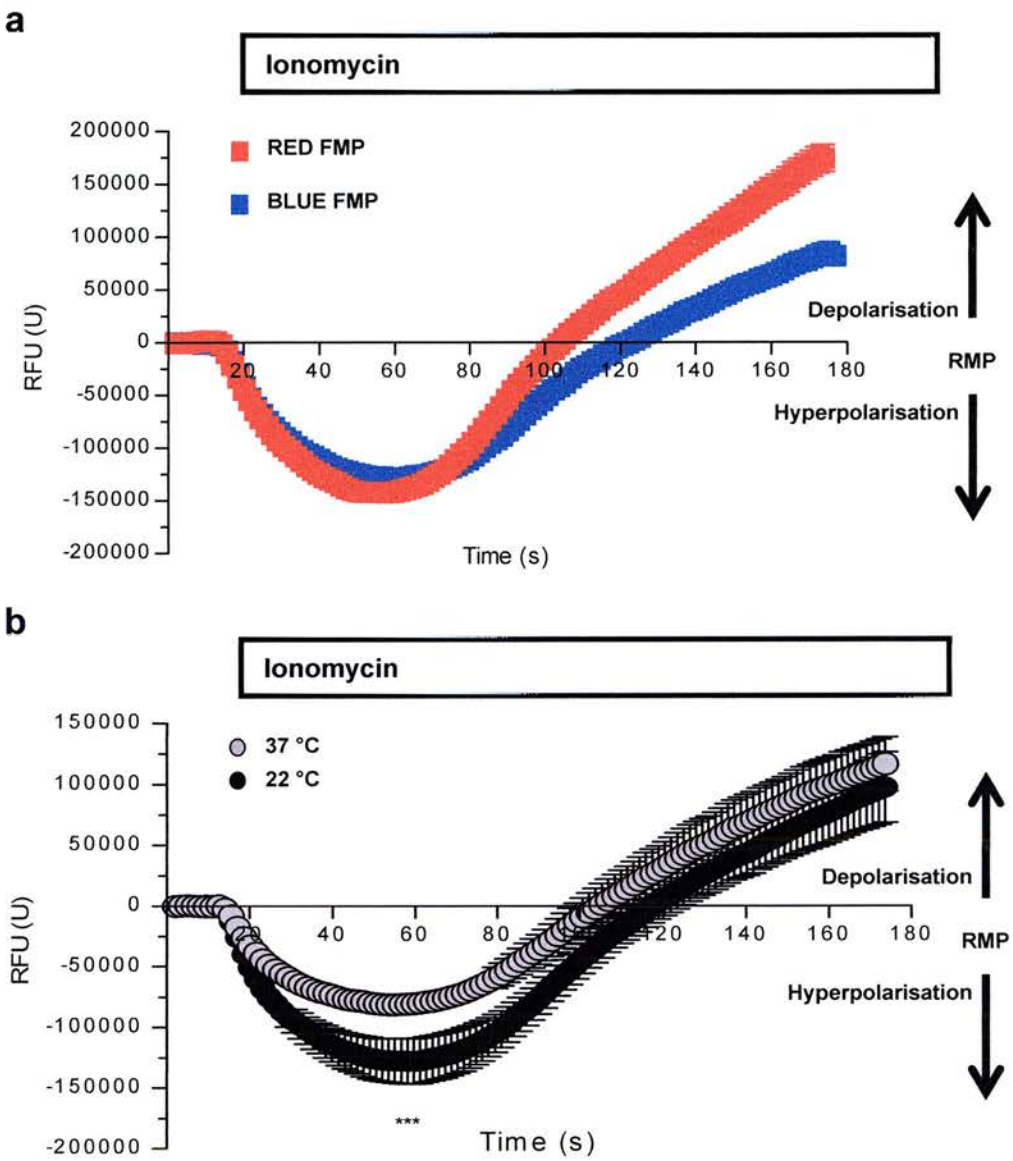


Figure 3.6 Ionomycin (1 μM) induced a significant prolonged hyperpolarisation in HEK293 cells transfected with the STREX variant.

a) The hyperpolarisation pattern and duration seen with both the RED and BLUE FMP dyes was the same. Data are mean ± s.e.m n=8.

b) The hyperpolarisation pattern and duration seen at 22°C and 37°C was significantly different when compared at the 70 second timepoint *** $p<0.001$ Students paired t-test. Data are mean ± s.e.m n=8.

Figure 3.7
Ionomycin induces hyperpolarisation as determined in whole cell patch clamp recordings

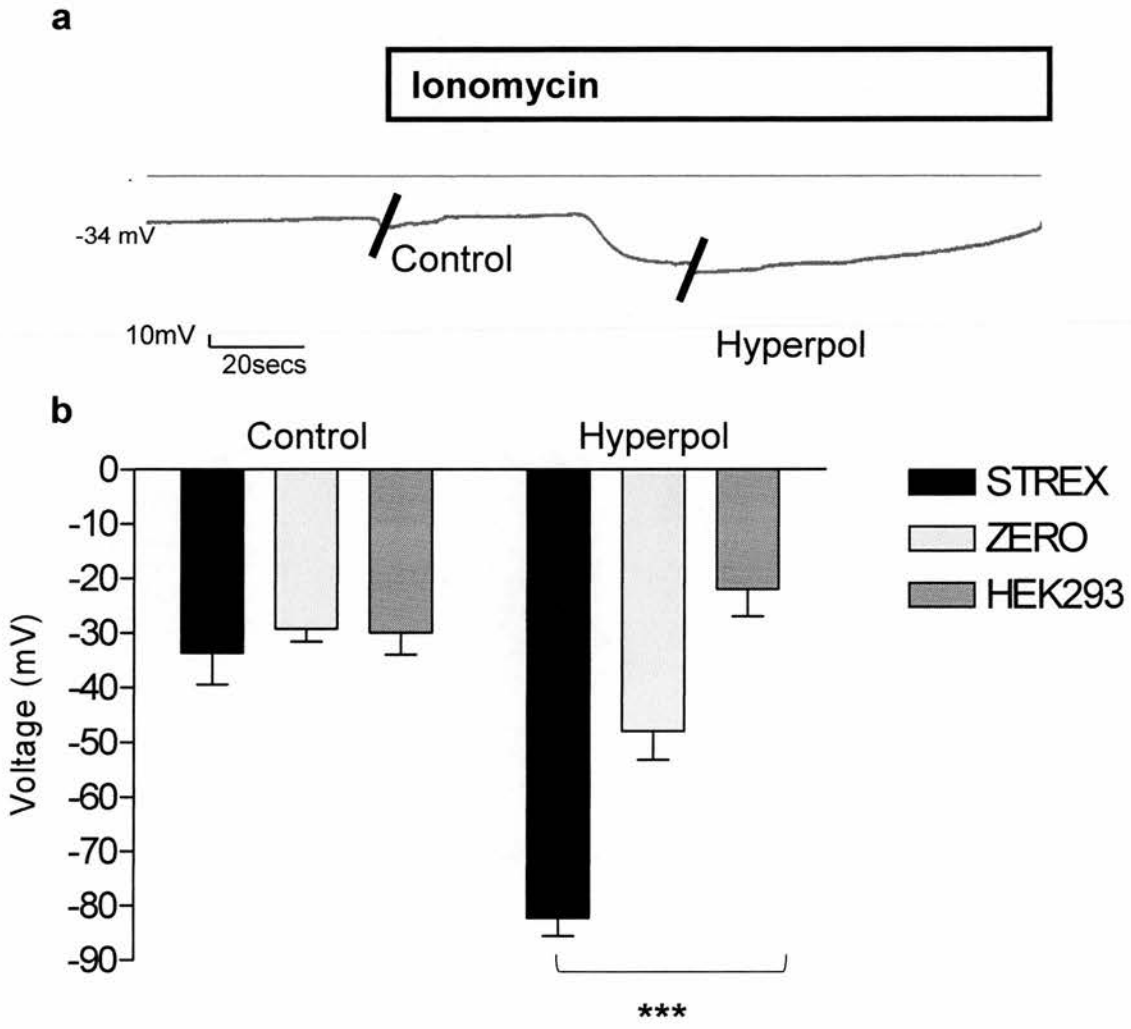


Figure 3.7 Ionomycin (1μM) was administered to cells in the whole cell current clamp configuration. a) shows an example recording of STREX with a baseline control membrane potential of -34 mV, the administration of ionomycin causes a clear hyperpolarisation followed by recovery depolarisation. b) Summary bar chart illustrates the control membrane potential of HEK293 cells alone, or expressing the ZERO or STREX BK channel variants and the change in membrane potential to 1 μM ionomycin administration taken at the 70 second timepoint. *** < p0.001 ANOVA with Tukey posthoc test comparing all responses to ionomycin. Data are mean ± s.e.m n>3.

(Data courtesy I.C.M.Rowe)

Another mode of confirmation that the hyperpolarisation effect seen in the FMP assay was caused by BK channel activity was by using the pharmacological agent paxilline, which is a selective inhibitor of BK channels (Knaus *et al.*, 1994b; Weaver *et al.*, 2006). After loading of the FMP dye, cells were preincubated for 10 minutes with 1 μM paxilline before the FMP assay was run. Upon ionomycin stimulation the paxilline pre-incubated STREX cells depolarised (Figure 3.8a), similar to the effect ionomycin has on untransfected HEK293 cells (Figure 3.3 and Figure 3.8b), thus verifying that the response to ionomycin was actually a BK specific response. Paxilline pre-incubation did not affect the ionomycin-induced depolarisation in untransfected HEK293 cells (Figure 3.8b).

The successful optimisation of the FMP assay allowed further investigation of BK channel splice variants and their differing apparent sensitivity to calcium. HEK293 cells were transiently transfected with the BK channel splice variant of choice STREX, ZERO or e22. Cells were seeded onto a 96-well plate and allowed to reach maximal cell confluency during 48 hours after which they were run on the optimised FMP assay protocol.

Figure 3.8
Paxilline blocks the ionomycin-induced hyperpolarisation in HEK293 cells expressing STREX channels

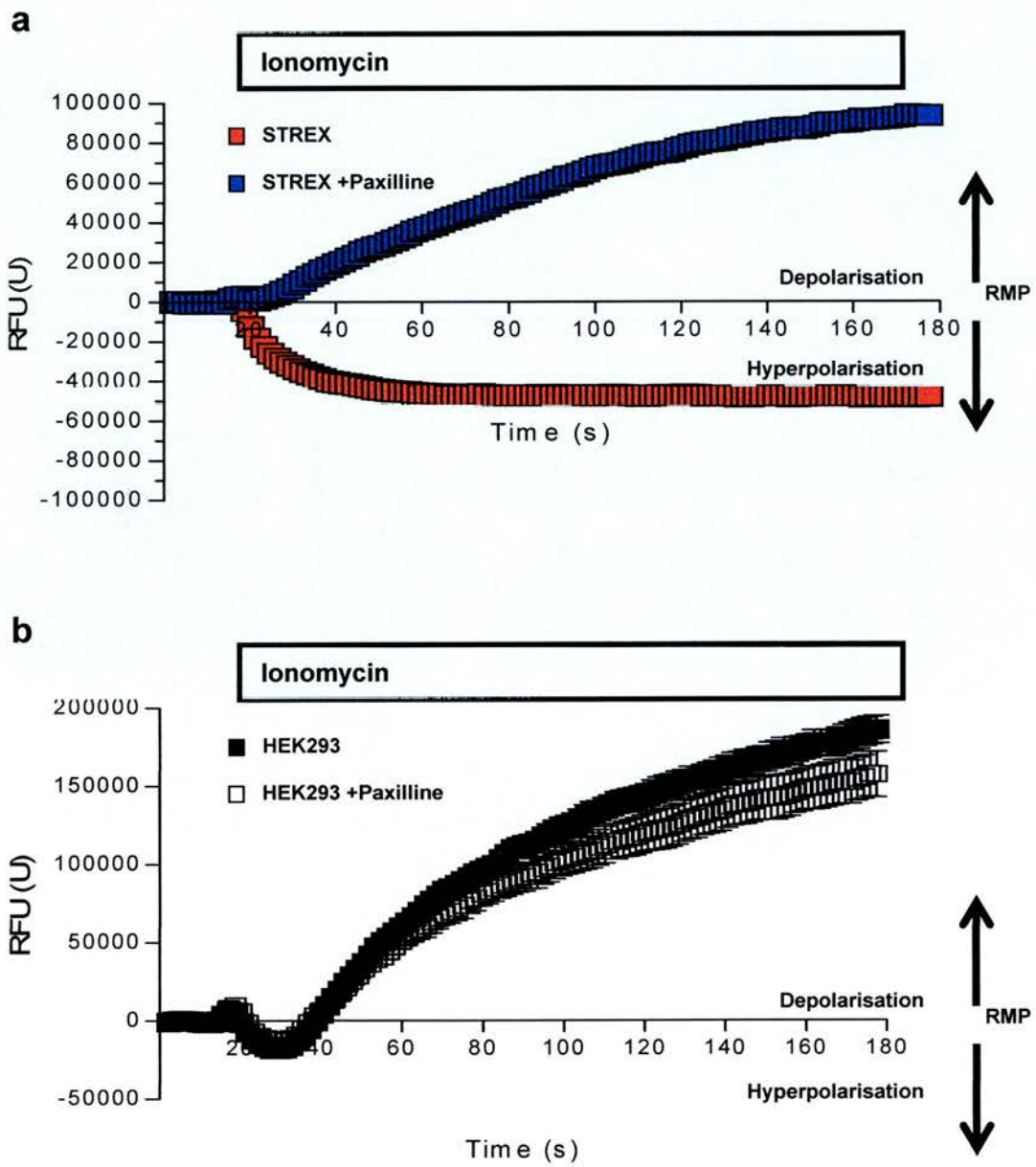


Figure 3.8 a) The ionomycin-induced hyperpolarisation in the STREX channel was completely blocked by the BK channel inhibitor paxilline (1 μM). b) Paxilline did not induce any significant changes in the HEK293 response to ionomycin. Data are mean ± s.e.m n=8.

In agreement with previous electrophysiological data (Erxleben *et al.*, 2002b; Chen *et al.*, 2005) BK channel splice variants showed different apparent calcium sensitivities in response to calcium influx. Cells expressing STREX channels showed the greatest hyperpolarisation (Figure 3.9), in comparison the e22 and ZERO BK variants which produced significantly smaller responses. The data obtained was analyzed at the 70 second time point, as this was considered the time point at which the maximal ionomycin-induced hyperpolarisation was observed in the FMP assay (Figure 3.3) and electrophysiology experiments (Figure 3.7). This was expressed as a percentage of the STREX response (taken as 100%), displaying the hierarchy in calcium selectivity by the variants e22 and ZERO (Figure 3.10, 64 ± 17 % and 45 ± 13 % respectively when compared to the STREX response taken as 100%, n= 8 $p<0.001$ ANOVA with Tukey post-hoc test). This data was in agreement with previously reported rank order (Chen *et al.*, 2005) in functional electrophysiology studies .

Figure 3.9
Discriminating between BK channel splice variants in the FMP assay

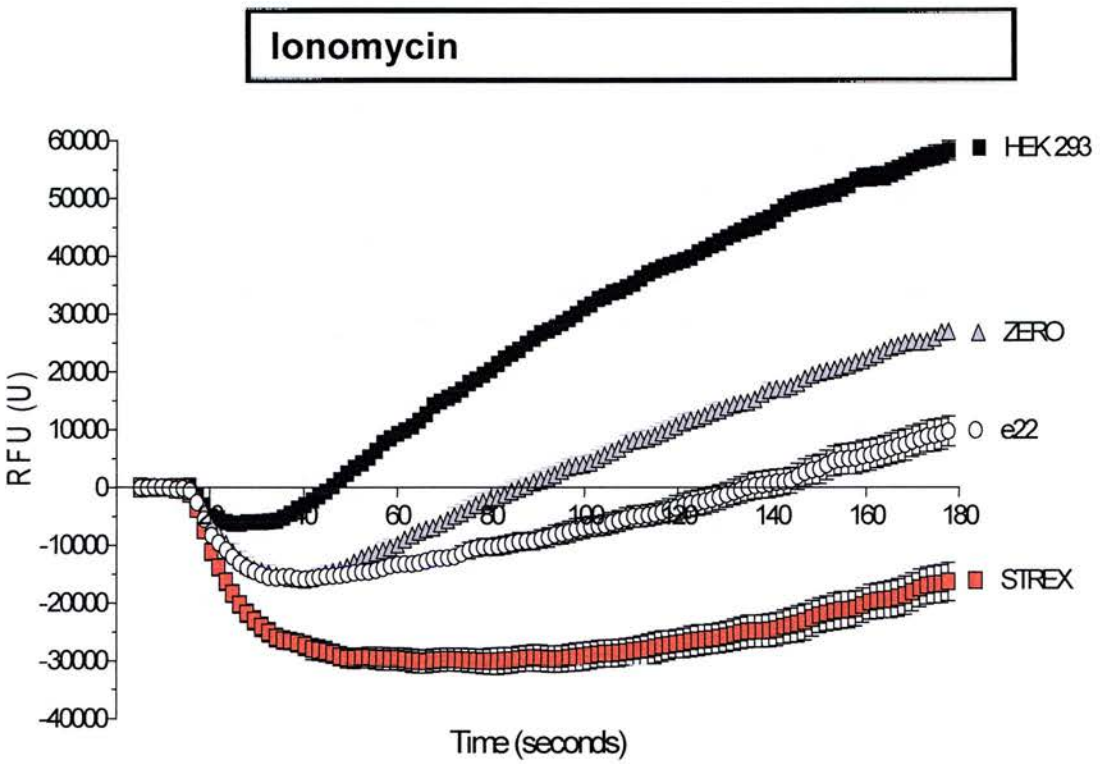


Figure 3.9 The STREX, ZERO, and e22 splice variants differ in the alternatively spliced inserts present at splice site 2 in the C-terminus of the BK channel α subunit and display a range of apparent calcium sensitivities. STREX shows a greater degree of ionomycin-induced hyperpolarisation than the e22 and ZERO variants, STREX > e22 > ZERO. Data are mean ± s.e.m n=8.

Figure 3.10
Discriminating between BK channel splice variants in the FMP assay

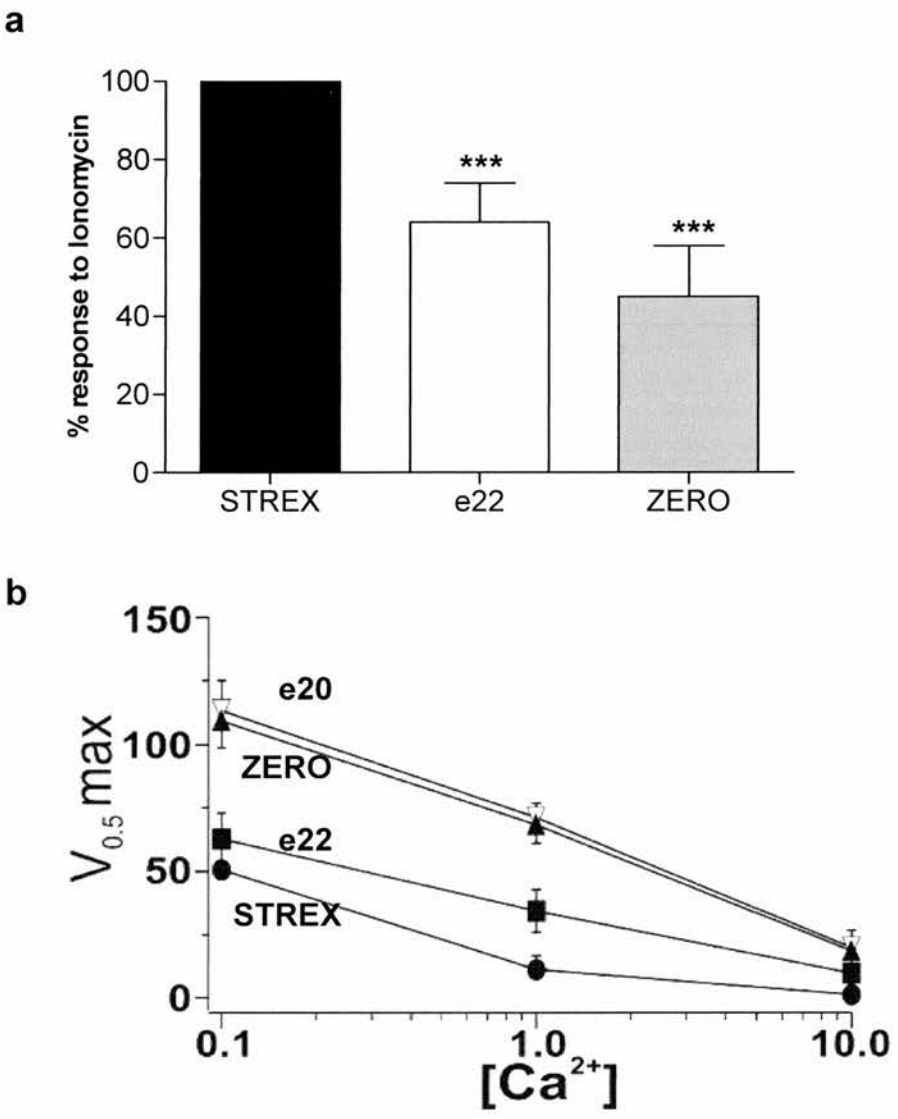


Figure 3.10 a) Ionomycin-induced hyperpolarisation of STREX, e22 and ZERO channels, at t = 70s as this was the average peak response time in experiments. e22 and ZERO produced significantly smaller responses, of 64 ± 17% and 45 ± 13% respectively, when compared to the STREX response (taken as 100%) (n=8, data are mean ± sem *** $p<0.001$ ANOVA with Tukey post-hoc test compared to STREX). b) In accordance with the rank order previously published, investigating the calcium sensitivity of the BK channel variants determined by patch clamp electrophysiology (see Chen *et al.*, 2005)

3.2.3 Screening of BK channel inhibitors

The BK channel splice variants have different apparent calcium sensitivities, so would be active under significantly different cellular conditions (Chen *et al.*, 2005). Therefore, it would be interesting to investigate the pharmacology of BK blockers to identify any differences in potency between the variants. Such differences could potentially be used to distinguish between variants expressed in native tissues and cell lines.

Paxilline (an indole diterpene alkaloid) was used as the specific BK inhibitor (Sanchez & McManus, 1996) during the initial optimization phase of the FMP assay, with a 1 μM concentration completely abolishing the ionomycin-induced hyperpolarisation induced by STREX expressed in HEK293 cells and was henceforth used as the negative control in all plates. Dose response experiments of paxilline in the FMP assay were obtained over a range of paxilline concentrations, a logarithmic increase in paxilline concentration decreased the ionomycin-induced hyperpolarisation illustrated by the STREX example shown (Figure 3.11a). Dose response curves were thus calculated using the Boltzmann dose-response equation for cells expressing all three splice variants; STREX, ZERO and e22 giving IC_{50} values of 0.35 ± 0.04 μM, 0.37 ± 0.03 μM and 0.70 ±

0.02 μM (Figure 3.11b) respectively, with the IC_{50} for the e22 variant being significantly decreased when compared to ZERO and STREX ($n = 4$, $p < 0.001$ F test).

The difference in the potency of paxilline then led to the investigation of other indole diterpene alkaloid inhibitors of BK channels, penitrem A and verruculogen (Knaus *et al.*, 1994a). Dose response curves were similarly obtained for penitrem A for all three splice variants. Cells expressing STREX, ZERO and e22 channels gave IC_{50} values of 0.08 ± 0.01 μM, 0.47 ± 0.20 μM and 2 ± 0.85 μM (Figure 3.12) respectively, with the IC_{50} for ZERO and e22 variants being statistically significantly different when compared to STREX ($n = 4$, $p < 0.001$ F test). Dose response curves of verruculogen were also obtained for the three splice variants. Cells expressing STREX, ZERO and e22 channels gave IC_{50} values of 8.6 ± 1.9 nM, 0.001 ± 1.7 nM and 0.001 ± 2.3 nM (Figure 3.13) respectively, with the IC_{50} for all variants being statistically significantly different to STREX ($n = 4$, $p < 0.001$ F test). Penitrem A and verruculogen did not induce any significant changes in the baseline activity of untransfected HEK293 cells (data not shown). The considerable differences in potency to verruculogen were surprising as these have not been previously reported, and

Figure 3.11
Dose response curves of BK channel variants to paxilline

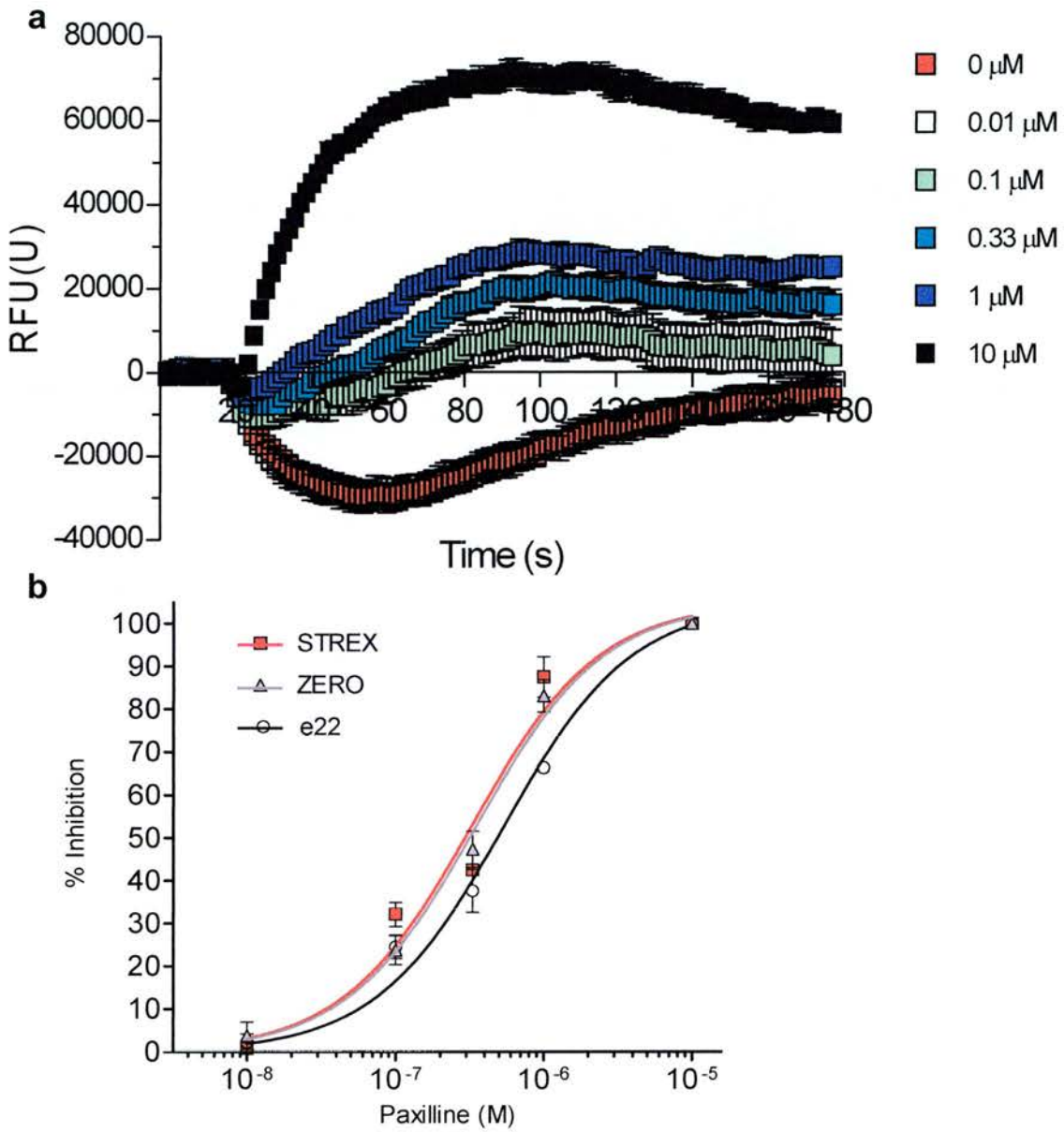


Figure 3.11 Dose response curves in the FMP assay were carried out for the BK channel specific blocker, paxilline, on all three variants a) representative experiment illustrates the decreasing ionomycin-induced hyperpolarisation of the STREX variant with increasing concentration of paxilline. b) The IC50 values obtained for all three splice variants channels STREX, ZERO and e22 gave values of 0.35 ± 0.04 μM, 0.37 ± 0.03 μM and 0.70 ± 0.02 μM (n=4) respectively, with the IC50 for the e22 variant statistically significantly different when compared to STREX and ZERO $p < 0.001$ F test.

Z' values to assess the assay quality are as follows for the IC50 plates: STREX = 0.87, e22 = 0.30 and ZERO = 0.61, values of > 0.30 are acceptable standards for assay reliability.

Figure 3.12
Dose response curves of BK channel variants to penitrem A

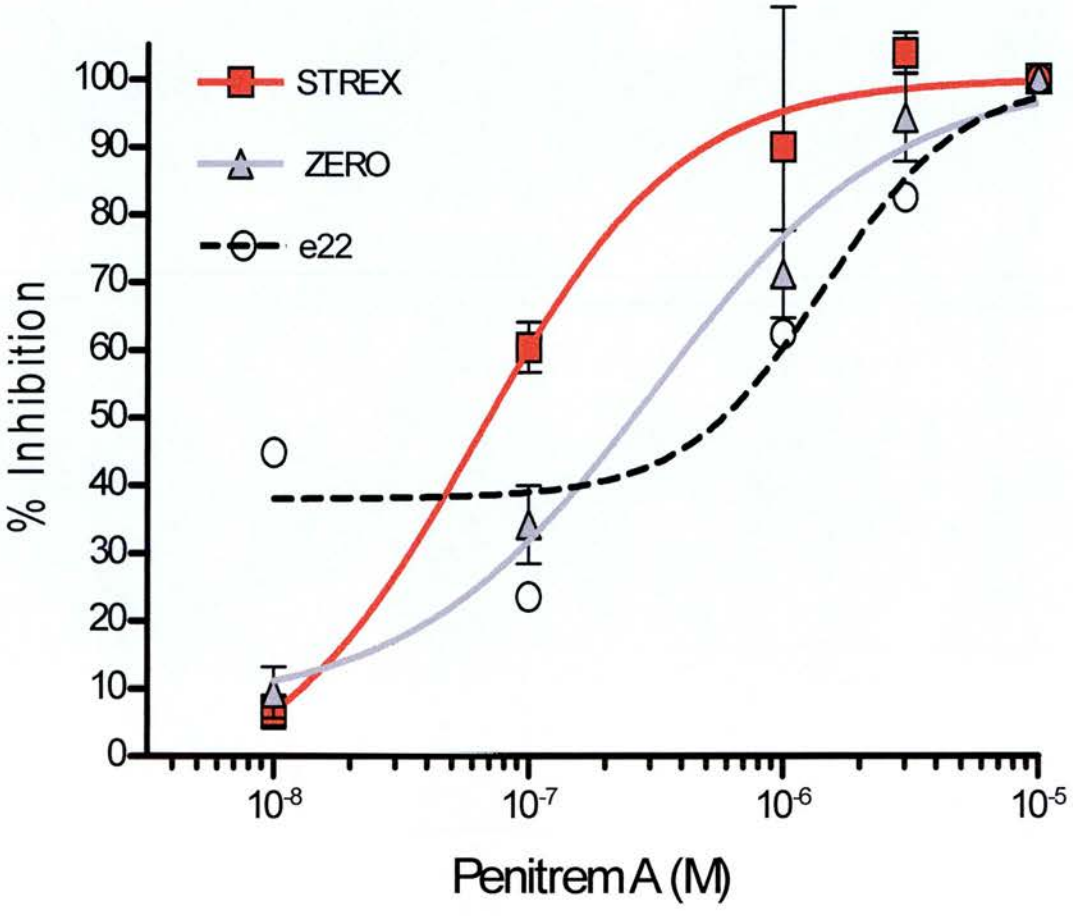


Figure 3.12 Dose response curves were obtained for all three splice variants STREX, ZERO and e22 in the FMP assay giving IC50 values of 0.08 + 0.01 μM, 0.47 ± 0.11 μM and 1.44 ± 0.1 μM (n=4) respectively, with the IC50 for the e22 and ZERO variants statistically significantly different when compared to STREX $p < 0.001$ F test.

Z' values to assess the assay quality are as follows for the IC50 plates: STREX = 0.45, e22 = 0.67 and ZERO = 0.53, values of > 0.30 are acceptable standards for assay reliability.

Figure 3.13
Verruculogen differentially blocks the ionomycin-induced hyperpolarisation of the BK channel splice variants

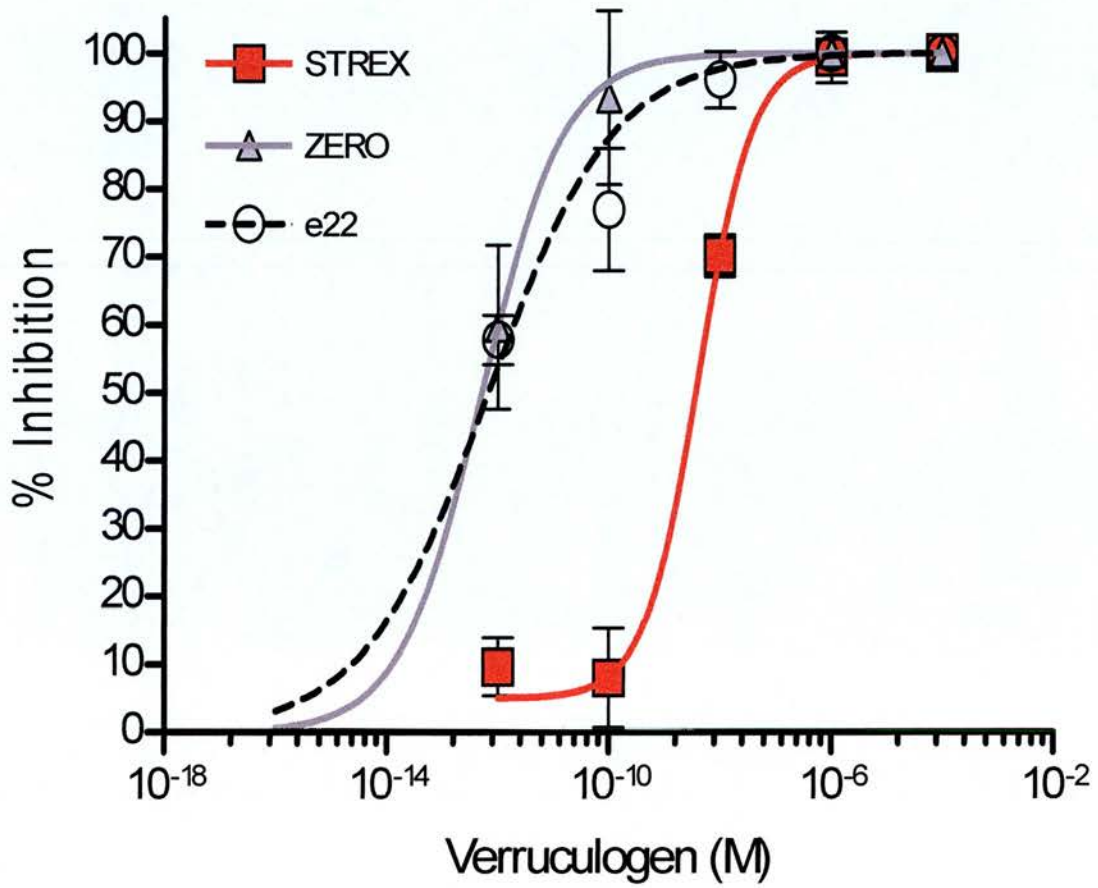


Figure 3.13 Dose response curves were obtained for all three splice variants STREX, ZERO and e22 in the FMP assay giving IC50 values of 5 ± 0.4 nM, 0.001 ± 0.03 nM and 0.001 ± 0.01 nM (n = 4) respectively, with the IC50 for the e22 and ZERO variants significantly different from STREX $p<0.001$ F test.

Z′ values to assess the assay quality are as follows for the IC50 plates: STREX = 0.88, e22 = 0.56 and ZERO = 0.83, values of > 0.30 are acceptable standards for assay reliability.

highlight the potential use of pharmacological agents in identifying different variants.

Tetraethylammonium (TEA) and the scorpion venom iberiotoxin are widely used pharmacological tools to inhibit BK channel activity. The potency of these two inhibitors on the three BK channel variants was also investigated in the FMP assay. The dose response assay format was similar to paxilline in that the wells were pre-incubated with the appropriate concentration of inhibitor prior to running the FMP assay. Although increasing TEA concentration attenuated the ionomycin-induced hyperpolarisation it failed to fully inhibit the response in any of the variants and produce a plateau in the dose response curve (Figure 3.14, n= 4). At the highest concentration of 10 mM TEA cells expressing STREX, ZERO and e22 channels elicited 41 ± 3 %, 53 ± 5 % and 29 ± 7 % inhibition respectively of the ionomycin-induced hyperpolarisation. However maximal concentrations of TEA had a significant depolarizing effect in non-transfected HEK293 cells.

Iberiotoxin also did not fully inhibit the ionomycin-induced hyperpolarisation response in any of the three variants in the FMP assay. Although iberiotoxin

inhibition did produce the characteristic sigmoidal curve, maximal concentrations of 1 μM inhibited the ionomycin-induced hyperpolarisation in cells expressing STREX, e22 and ZERO channels by 28 ± 2 %, 72 ± 5 % and 65 ± 11 % (Figure 3.15). Iberiotoxin inhibition of the e22 and ZERO ionomycin-induced hyperpolarisation was significantly higher to that of STREX (n = 4, $p<0.001$ F-test). e22 had a greater sensitivity to iberiotoxin as even 0.1 nM of iberiotoxin caused a 24 ± 9 % inhibition of the ionomycin-induced hyperpolarisation, whereas STREX appeared to be the least sensitive in this assay. Iberiotoxin pre-incubation did not affect the baseline of untransfected HEK293 cells.

3.2.4 Ionomycin-induced hyperpolarisation of all STREX cysteine mutant channels in the FMP assay

It has previously been investigated in electrophysiology assays that the cysteine residues C23 and C25 within the STREX variant contribute to its apparent high calcium sensitivity (McCartney *et al.*, 2005). Hence using site-directed mutagenesis the six cysteine residues within STREX were mutated to alanine (C12A, C13A, C12:13A, C16A, C23A, C25A, C23:25A and C51A) to investigate the ionomycin-induced hyperpolarisation and thus changes in the apparent

calcium sensitivity of the channel. The FMP assay was run on the cysteine mutants, alongside STREX and ZERO as these two BK variants have known high and low sensitivities respectively to ionomycin stimulation (Figure 3.10). The mutants all displayed different degrees of ionomycin-induced hyperpolarisation indicating their potential role in the increased calcium sensitivity of STREX.

As with previous FMP assay plates data points were compared at the t = 70 seconds timepoint, ZERO, C12A, C13A and C12:13A produced significantly smaller responses, of 18 ± 22%, 5 ± 3% 57 ± 9%, 74 ± 4% respectively, when compared to the STREX response (taken as 100%) (Figure 3.16, N = 3, n = 12, data are mean ± sem * $p<0.05$ & *** $p<0.001$ ANOVA with Tukey post-hoc test). Thus the FMP assay suggested that the high apparent calcium sensitivity of STREX may be largely determined by the C12 and C13 residues within the cysteine rich insert

Figure 3.14
Dose response curves of BK channel variants to TEA

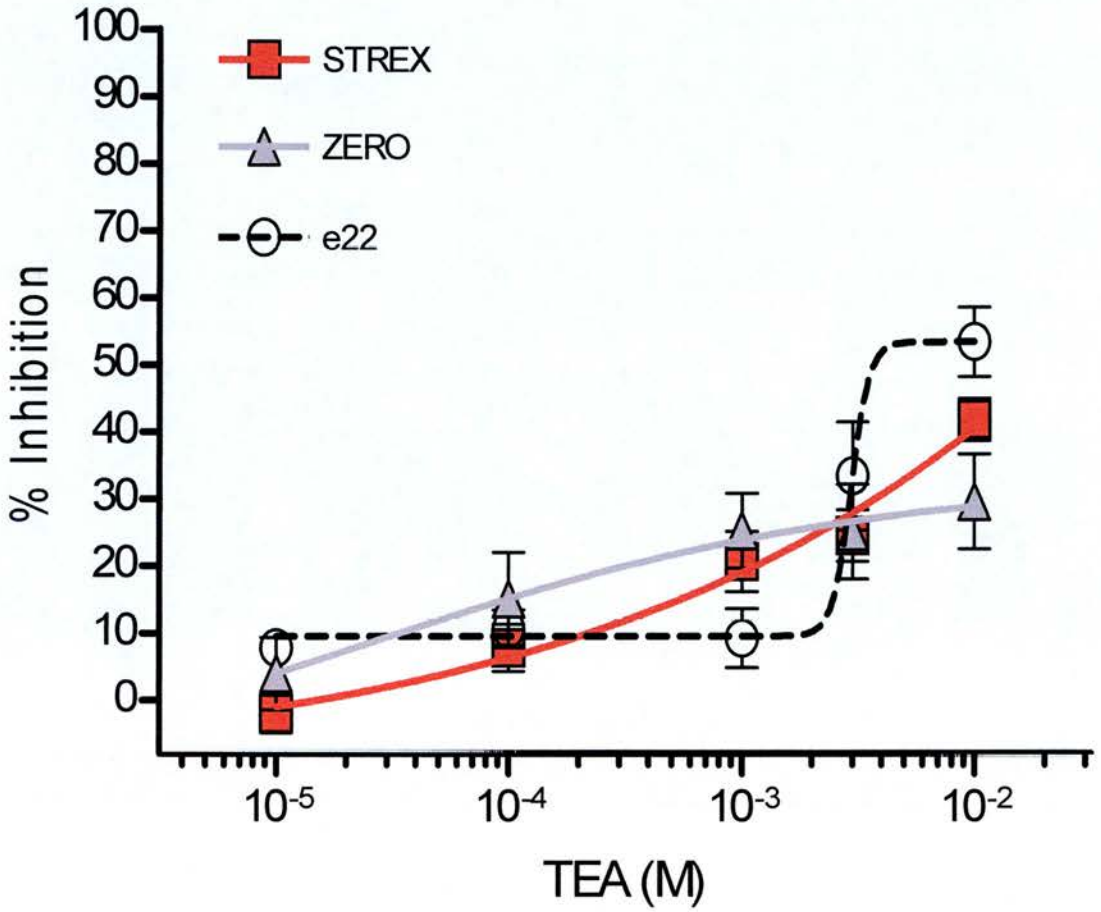


Figure 3.14 Dose response curves were obtained for the STREX, ZERO and e22 variants (n=4). 100% inhibition of the ionomycin-induced hyperpolarisation was not achieved in the FMP assay.

Z' values to assess the assay quality are as follows for the IC50 plates: STREX = 0.70, e22 = 0.45 and ZERO = 0.63, values of > 0.30 are acceptable standards for assay reliability.

Figure 3.15
Dose response curves of BK variants to iberiotoxin

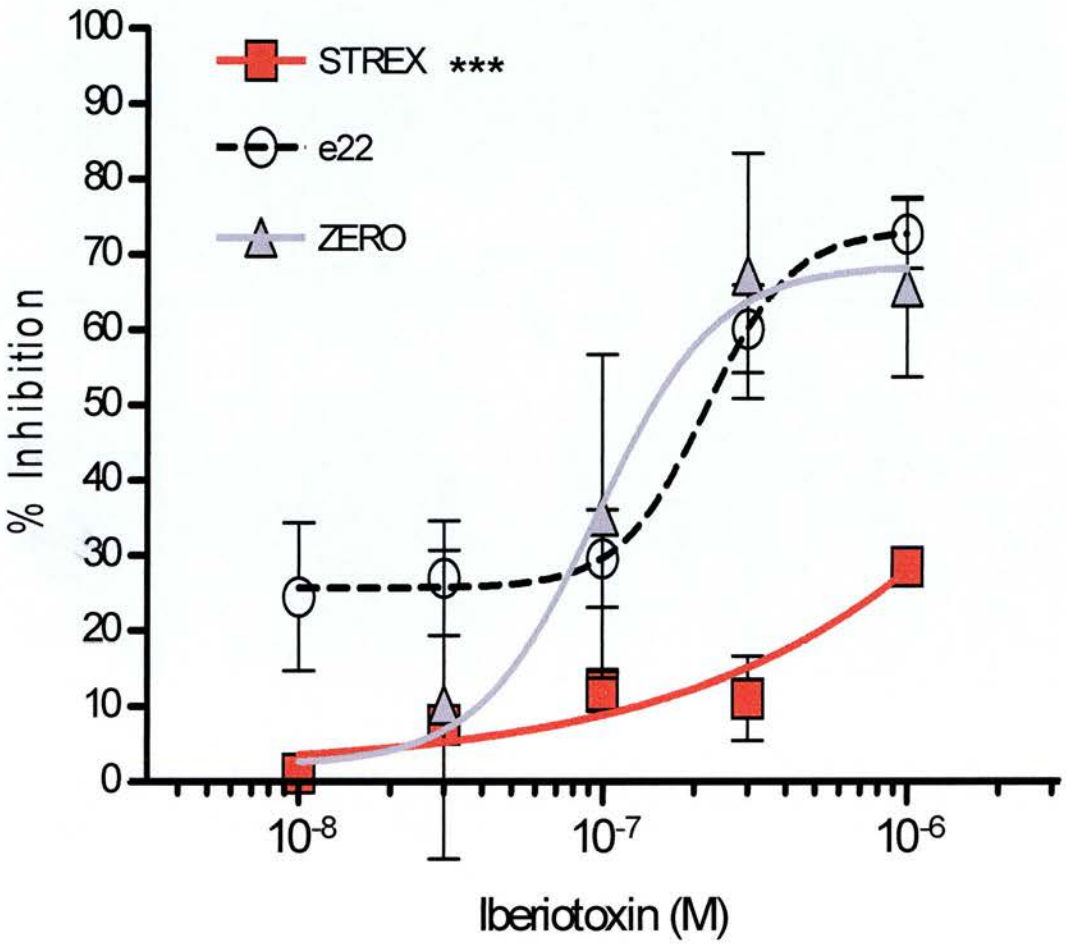


Figure 3.15 Dose response curves were obtained for the STREX, ZERO and e22 variants (n=4). The e22 and ZERO response was significantly different to STREX *** p> 0.001 F-test but 100% inhibition of the ionomycin-induced hyperpolarisation was not achieved for any variant in the FMP assay.

Z' values to assess the assay quality are as follows for the IC50 plates: STREX = 0.55, e22 = 0.46 and ZERO = 0.43, values of > 0.30 are acceptable standards for assay reliability.

Figure 3.16
Ionomycin-induced hyperpolarisation of all STREX cysteine mutant channels in the FMP assay

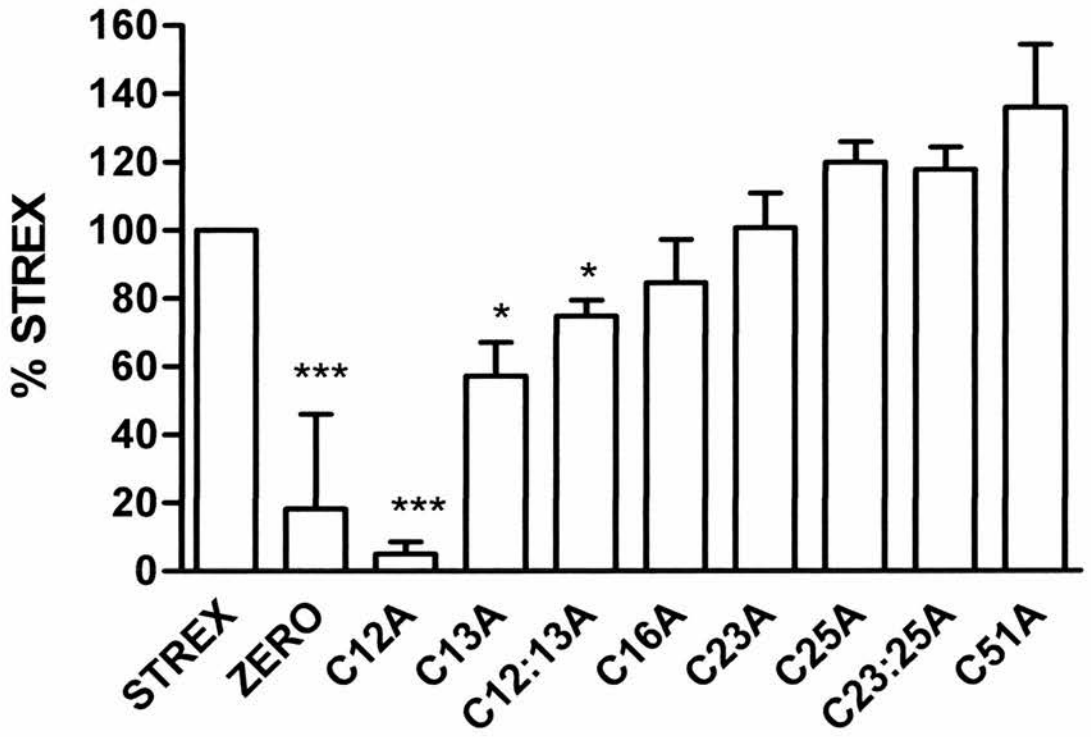


Figure 3.16 Summary bar chart shows the ionomycin-induced hyperpolarisation of HEK293 cells expressing STREX cysteine mutant channels in the FMP assay, expressed as a percentage of the STREX channels response. Mutating the six cysteine residues in STREX to alanine changes the activation of the channel, in response to the ionomycin mediated calcium influx.

Data points were compared at the t = 70s. ZERO, C12A, C13A and C12:13A produced significantly smaller responses, of 18 ± 22%, 5 ± 3% 57 ± 9%, 74 ± 4% respectively, when compared to the STREX response (taken as 100%) (N = 3, n = 12, data are mean ± sem * $p<0.05$ & *** $p<0.001$ ANOVA with Tukey post-hoc test).

3.3 Summary

Here I have successfully developed and optimised for the first time a high-throughput voltage sensitive fluorescent assay (FMP) on FlexStation® II to screen BK channel variants, which is robust and reproducible. Previous physiological and pharmacological characterisation of BK channel modulators has depended on using low-throughput methods such as ligand binding or electrophysiology. The robustness and ease of the FMP assay renders it an excellent high-throughput preliminary screen for isolating potentially therapeutic drugs for BK channels and investigating the functional channel properties. Therefore only interesting drugs or properties of channels are taken forward and investigated at single channel level in patch clamp electrophysiology, as the FMP assay has limitations in that a population of channel expressing cells are observed and thereby providing little detail on channel kinetics. Nonetheless voltage sensitive dyes are an excellent tool to initially investigate the functional effects and interactions of channels.

In agreement with previously published work (Chen *et al.*, 2005) I have been able to show that the variants have different sensitivities to ionomycin mediated calcium influx, with STREX having higher calcium sensitivity and thereby

having a greater ionomycin-induced hyperpolarisation response (STREX > e22 > ZERO). This highlights the potential use of the FMP assay to investigate not only other splice variants of BK, but to use the assay to probe the association of different β subunits and their splice variants co-expressed with the different splice variants of BK α.

Investigation into the role of the cysteine residues in the apparent increased calcium sensitivity of STREX channels, showed that in fact the C12 and C13 were important in mediating the ionomycin-induced hyperpolarisation in this assay, this will be discussed further in light of electrophysiological data in Section 4.

Pharmacological analysis of the variants revealed that verruculogen could potentially be used as a tool to differentiate between the variants in native cells and tissues expressing BK channels, as e22 and ZERO displayed significantly higher potency for the inhibitor than STREX (Table 3.1). Paxilline and penitrem A had a sub-micromolar potency on BK, whereas verruculogen had a sub-nanomolar range with distinct significant differences between the STREX and e22 or ZERO BK variants tested.

Table 3.1
Comparison of known BK channel pharmacology

Inhibitor	Published		IC50 FMP Assay		
	IC50	Kd [1]	STREX	ZERO	e22
Paxilline	17nM	170nM	0.35 ±0.04 μM	0.37 ±0.03 μM	0.70 ±0.02 μM
Penitrem A	17nM	1700nM	0.08 ±0.01 μM	0.47 ±0.11 μM	1.44 ±0.01 μM
Verruculogen		170nM	5 ±0.4 nM	0.001 ±0.03 nM	0.001 ±0.03 nM
TEA [4]	0.5mM		na	na	na
Iberiotoxin [3]	9.7nM		na	na	na
Charybdotoxin [2]	0.8nM		--	--	--

1. Knaus *et al.,* 1994b; bovine aortic smooth muscle
2. Miller *et al.,* 1985; rat skeletal muscle
3. Galvez *et al.,* 1990; bovine aortic smooth muscle
4. Kang *et al.,* 1996; rat pyramidal neurons

Though the exact values for the IC50's reported here with the FMP assay differ from those previously reported, this is likely to be attributed to the different methods which are used to obtain IC50 values. The Kd data that is published on the indole diterpene alkaloids, paxilline, penitrem A and verruculogen is via binding assays on BK competing with radioactive charybdotoxin. Interestingly though paxilline and verruculogen had similar binding properties to BK but verruculogen had greater potency in electrophysiological experiments in bovine smooth muscle (Knaus *et al.*, 1994b).

Paxilline (Sanchez & McManus, 1996), penitrem A and verruculogen (Knaus *et al.*, 1994b) are thought to mediate their inhibition via allosteric interactions with the pore that may alter the shape of the pore hence inhibiting K^+ efflux. Paxilline has been reported to have allosteric but not competitive, interactions with calcium as increasing calcium concentrations decreased the degree of inhibition by paxilline (Sanchez & McManus, 1996). STREX has higher calcium sensitivity than ZERO or e22, so it could be that the structural difference in STREX that confers this increased apparent calcium sensitivity may significantly alter the channel structure thus inhibiting the pore which results in the

decreased potency of STREX to verruculogen in comparison to the other two variants.

TEA and iberiotoxin did not fully abolish the ionomycin-induced hyperpolarisation in the FMP assay, suggesting that these inhibitors are not as potent in this assay, as they are known to efficiently block BK channel activity in electrophysiological studies. TEA mediates a "flickery block" of the channel, as it is thought to sit in the pore of the channel and therefore reduce channel opening (Kang *et al.*, 1996). It could be that the ionomycin stimulated influx of calcium ions in a population of cells, as investigated here in the FMP assay, is such that due to the activity of a few open channels there is enough whole cell current present to elicit significant ionomycin-induced hyperpolarisation. It has been reported that 10 mM TEA does not fully inhibit voltage-activated potassium channel activity but that increasing the concentration to as high as 50mM does achieve full block (Farley & Rudy, 1988). However TEA can affect the activity of other channels, as well as inducing HEK293 cell effects at such concentrations and loses BK channel specificity at > 1 mM.

Iberiotoxin is thought to have similar mode of action of BK channel blockage to TEA, by interacting with the P-region of the channel pore it effectively "plugs" the channel (Candia *et al.*, 1992). STREX channels were least sensitive to iberiotoxin inhibition in the FMP assay; again this could be due to its increased sensitivity to calcium. It is reported that a 10 μM concentration of ionomycin allows an influx of calcium up to 1 μM in HEK293 cells (Ribeiro *et al.*, 2000). However this would be enough to cause hyperpolarisation in some transfected cells as the $V_{1/2}$ max of STREX is significantly lower to that of the other variants hence requires less stimulus to be activated (Chen *et al.*, 2005).

It could therefore be possible that the STREX insert is behaving like the β4 subunit, which when associated to the BK α subunit prevents the iberiotoxin block of BK channels as shown by binding studies and outside-out patching of *Xenopus oocytes* (Meera *et al.*, 2000). Thereby these data suggest that inclusion of the STREX insert changes the structure of the channel and somehow modifies the outer vestibule of the channel pore.

Chapter Four: Role of cysteine residues in the STREX insert in the control of channel sensitivity to hypoxia

4.1 Introduction

Oxygen deprivation can produce severe clinical conditions, for instance myocardial infarction and stroke, in tissues that are dependent on aerobic metabolism such as the myocardium, central nervous system and kidneys. Sensing changes in oxygen tension is a vital requirement for mammalian cell survival, due to the central role of oxygen as an acceptor of electrons in the mitochondrial respiratory chain allowing the production of ATP, by oxidative phosphorylation, to sustain highly active processes. To protect against adverse effects during episodes of oxygen deprivation (hypoxia) and to maintain biological homeostasis cells require an adequate oxygen supply or the ability to rapidly detect and respond to changes in oxygen tension, ensuring that cellular function is not compromised (Lopez-Barneo *et al.*, 1997).

Acute hypoxia is known to trigger rapid respiratory and cardiovascular changes in the mammalian body to ensure sufficient oxygen supply to the critical organs such as the brain and heart. Oxygen sensitive ion channels are thought to be major effectors of cellular responses to hypoxia and are found to be expressed in excitable neurosecretory cells (Lopez-Barneo *et al.*, 2004). BK channels are

important for detecting changes in oxygen tension in specialized tissue such as the carotid body or epithelia (Riesco-Fagundo *et al.*, 2001; Jovanovic *et al.*, 2003), as well as having a role in controlling membrane excitabililty in vascular smooth muscle and neurons (Liu *et al.*, 1999; Porter *et al.*, 2001). BK channel responses to changes in oxygen tension have been shown to regulate pulmonary artery tone, and implement responses such as hypoxic pulmonary vasoconstriction (Weir *et al.*, 2005) and hypoxic foetoplacental vasoconstriction to improve ventilation-perfusion matching (Hampl *et al.*, 2002). Conversely sensitivity of BK channels to oxygen is as diverse as the tissues that express them, as some are potently inhibited by hypoxia (Riesco-Fagundo *et al.*, 2001; Lewis *et al.*, 2002; Jovanovic *et al.*, 2003) whilst others are non-responsive (Park *et al.*, 1995; Wyatt & Peers, 1995).

As discussed previously in Section 1.5 hypoxia induces a very complex physiological response and the role of BK channels within it is widely disputed. At present there are three main theories on the hypoxic modulation of BK channels: a) the heme-oxygenase (HO2) (Williams *et al.*, 2004), b) AMP kinase (Wyatt *et al.*, 2007) and c) STREX splice variant selectivity (McCartney *et al.*, 2005). Co-expression of the BK channel α subunits with β1 subunits in HEK293

cells produces channels that are potently inhibited by hypoxia, a mechanism which is dependent on carbon monoxide and heme-oxygenase (HO2) (Williams *et al.*, 2004) . The expression of BK channel β1 subunits is however largely restricted to vascular smooth muscle cells. Therefore whilst the proposed mechanism could be applied to these cells it can not solely explain the sensitivity of BK channels to hypoxia which is observed in numerous other tissues in which β1 subunits are not expressed (Liu *et al.*, 1999). Thus it remains to be seen if the HO2 mechanism applies to other BK channels which are expressed as α tetramers alone or indeed associated with other β subunits, as it is possible that these may display differential sensitivity to hypoxic conditions. The AMP activated kinase theory proposes that hypoxic inhibition of oxidative phosphorylation induces activation of the AMP kinase leading to inhibition of BK channels (Wyatt *et al.*, 2007). Although this mechanism shows co-localisation of the AMP kinase and BK channel there is no membrane delimited data suggesting an absolute requirement of an intact cytosolic network.

Investigations into the role of different splice variants of BK in acute hypoxia in excised inside-out patches demonstrated that oxygen sensing by the α subunits of BK channels was conferred by the cysteine rich STREX splice variant insert, as

the ZERO variant channel activity was unaffected by changes in oxygen tension (McCartney *et al.*, 2005). These data suggested that the differential hypoxic sensitivity of the channel in different tissues could be dependent on splice variant modulation. Evolutionary conserved cysteine residues are encoded within the STREX insert and a -CSC- ($C_{23}S_{24}C_{25}$) motif that is highly conserved from fish to man has been identified (Figure 4.1). This motif has also been identified in other oxygen sensitive channels; TASK1 (O'Kelly *et al.*, 1999; Lewis *et al.*, 2001), whilst is absent in the closely related but oxygen insensitive TASK2 channel (Lewis *et al.*, 2001). Site-directed mutagenesis of this motif (C23:25A and S24A) abolished the hypoxia sensitivity of the STREX variant. Thus, a structural motif incorporating a serine residue flanked by a cysteine residue may be important for sensitivity of BK channels to hypoxia (McCartney *et al.*, 2005). It is therefore important to decipher a direct or intrinsic mechanism for hypoxic regulation of STREX channels, thus placing it within the hierarchy of diverse control signals triggered by hypoxia.

Figure 4.1
Identification of cysteine residues within STREX insert.

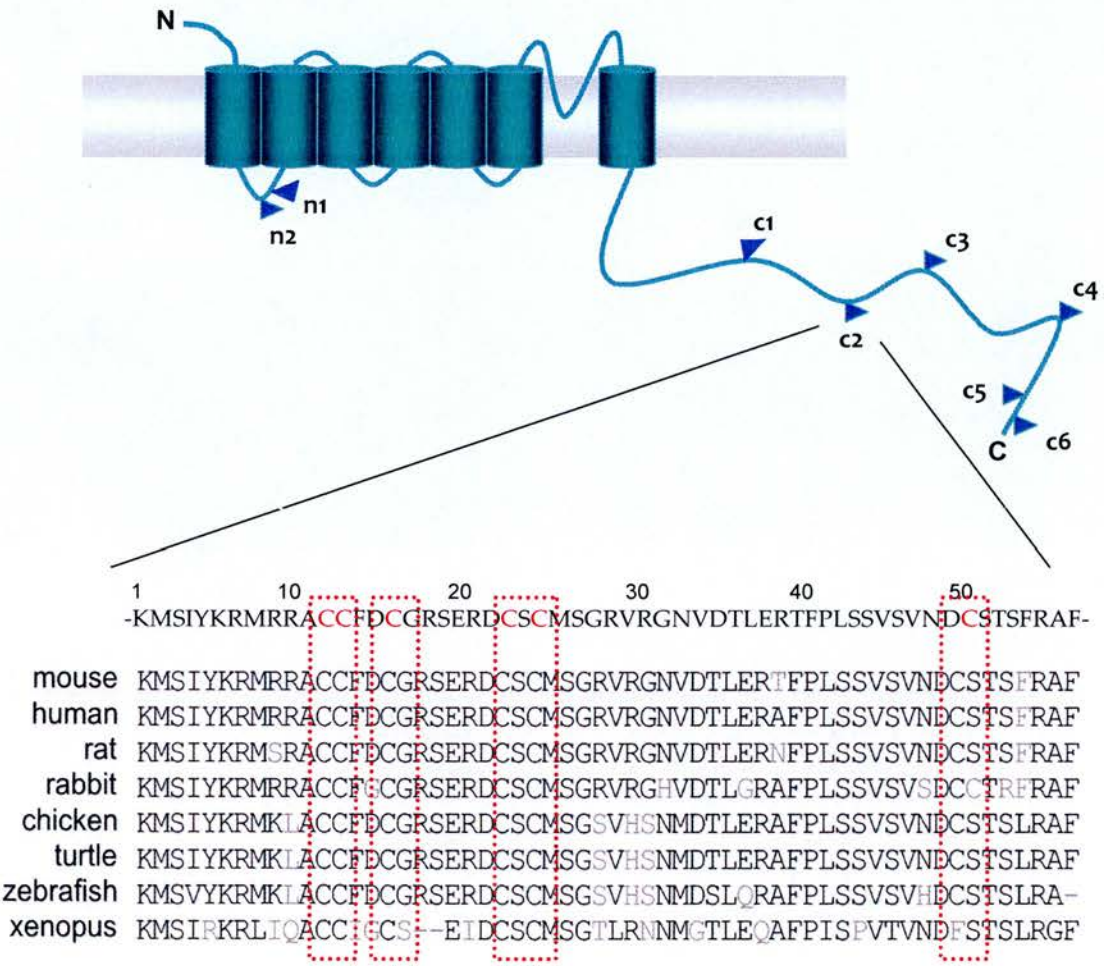


Figure 4.1 Evolutionary conserved cysteine residues in the 59 amino acid STREX insert

The thiol groups (-SH) of cysteine amino acids are readily polarisable and the weakness of the S-H bond means the sulphur atom can be stabilised by sharing an electron with an adjacent atom. Thereby allowing two highly reactive vicinal cysteine residues to interact and form disulphide bonds to stabilise protein structure. Serine residues adjacent to cysteines are thought to modify the local environment promoting disulphide bond formation (Salmeen *et al.*, 2003) in protein structures. The STREX insert contains a motif where another cysteine and serine residue are adjacently located (C51 and S52, Figure 4.1), these could potentially behave in a similar fashion to the proposed -CSC- hypoxia sensing motif by mediating disulphide bonds with vicinal residues.

Hypoxia could induce conformational changes which may disrupt potential inter-subunit disulphide bonds mediated by the individual cysteine residues within the STREX insert itself or other cysteine residues within a single α subunit, as cysteine-cysteine interactions in other proteins are sensitive to reversible oxidative regulation (Park *et al.*, 1999; Kurek *et al.*, 2002; Tang *et al.*, 2004). Cysteine interactions could potentially exist between different α subunits, therefore disruption of intra-subunit disulphide bonds could contribute to the STREX hypoxic response. To investigate this hypothesis

further, patch clamp electrophysiology and site-directed mutagenesis was utilised to generate mutants of cysteine residues located immediately up or downstream of the -CSC- motif within the STREX insert, where the cysteines were mutated to alanine.

4.2 Results

4.2.1 Effects of cysteine mutations on the response of STREX channels to hypoxia

As shown previously (McCartney *et al.*, 2005) acute exposure to hypoxia resulted in a reversible inhibition of STREX channel activity in isolated inside-out patches from transiently transfected HEK293 cells, in the absence of exogenous intracellular metabolites under symmetrical potassium gradients at +40 mV and in the presence of 0.1 μM intracellular free calcium (Figure 4.2).

Under these conditions STREX single channel open probability (Po) during the normoxic stabilisation phase directly after excision of the patch was stable and did not vary significantly from patch-to-patch, with a range of 0.3 - 0.6. Upon perfusion of the intracellular face of the patch with hypoxic solution (pO_2 <

20mm/Hg) a rapid and sustained significant decrease in Po was observed (mean % inhibition was 60 ± 9 % , n = 13, $p<0.001$ Kruskal-Wallis test with Dunns posthoc compared to respective normoxic control) that reversed upon washout with normoxic solution (Figure 4.2).

Site-directed mutagenesis to mutate all cysteines residues within the STREX insert into alanines was used as a direct approach to elucidate the potential role of STREX cysteine residues in the response of STREX channels to hypoxia. Six different mutant constructs of STREX were thus created; C12A, C13A, C16A, C23A, C25A, C23:25A and C51A (Figure 4.1). Section 3.2.3 demonstrated the different ionomycin-induced hyperpolarisation responses these mutants had in the FMP assay, which suggested that C12 and C13 played a significant role in the calcium sensitivity of STREX, as mutation of these residues to alanine greatly reduced the ability of STREX to respond to an increase in calcium influx. As STREX channel sensitivity to hypoxia is calcium sensitive, not only the previously highlighted C23 and C25 residues but in fact the C12 and C13 amino acids could also have a role in the STREX hypoxia response. Thus, by mutating C12 and C13 we hypothesised that the STREX hypoxia response would be abolished.

Figure 4.2
The response of STREX channels to hypoxia

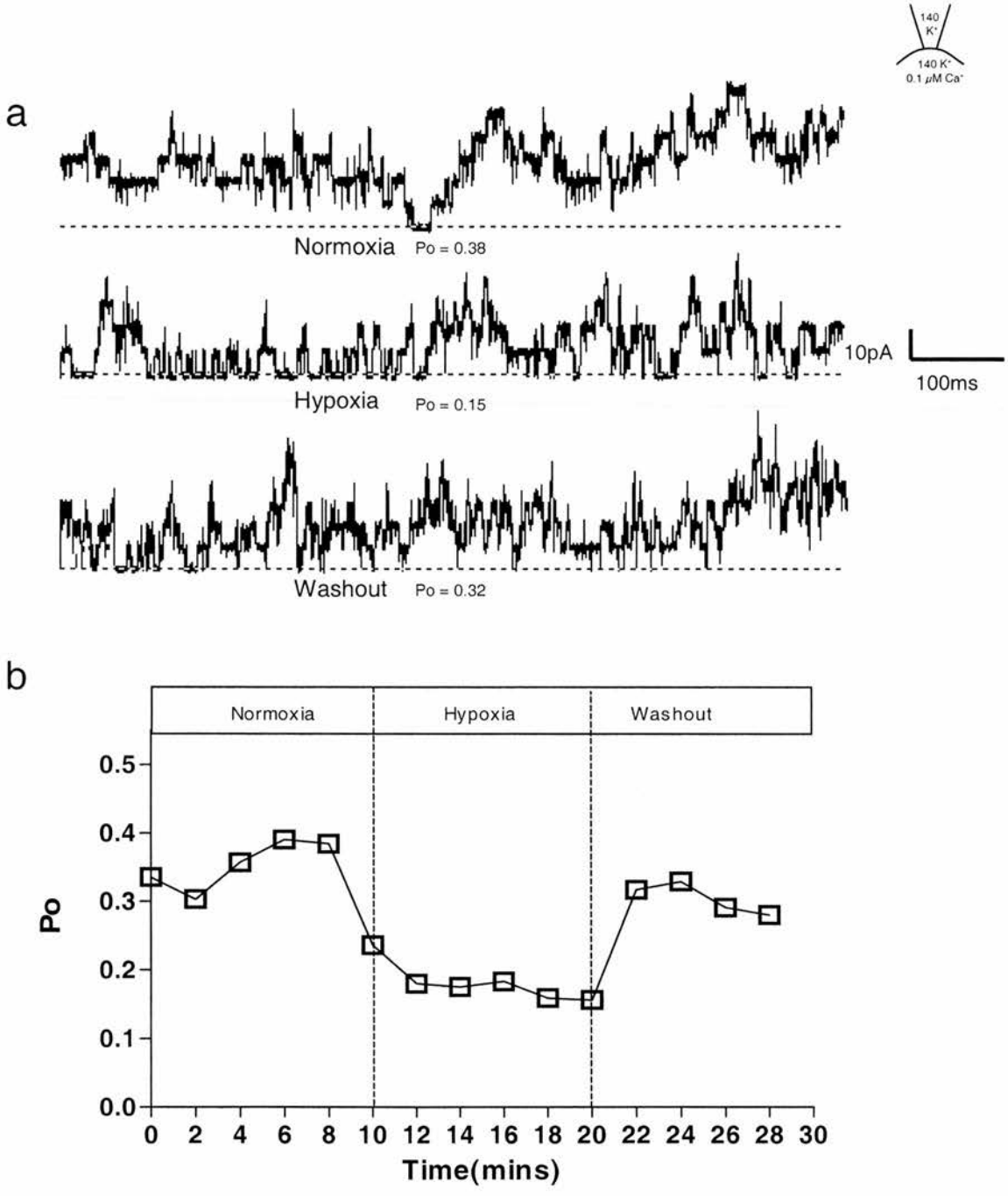


Figure 4.2 Example recordings of single channel activity in an excised inside-out patch, of STREX channels transiently expressed in HEK293 cells at 40mV. Panel a shows control recordings in normoxic solution, Po values are indicated. Hypoxia induced a significant decrease in channel activity that was reversed upon washout with normoxic solution. The time course of the response from the patch in panel a is illustrated in panel b.

The mutations produced functional channels that were efficiently expressed in HEK293 cells and had no significant difference in single channel amplitude when compared to STREX control (at +40 mV unitary current amplitude was 9.7 ± 0.14 pA, 9.5 ± 0.11 pA, 9.7 ± 0.30pA, 9.38 ± 0.13pA, 9 ± 0.20 pA, 9.8 ± 0.20 pA, 9.49 ± 0.08 pA and 9.86 ± 0.16 pA, for STREX wildtype, STREX-C12A, STREX-C13A, STREX-C16A, STREX-C23:25A, STREX-C23A, STREX-C25A, and STREX-C51A respectively, $n > 3$).

There were significant differences in the starting Po during the control normoxic period, straight after excision of the patch between several of the mutants in comparison to the STREX controls conducted in parallel. These were STREX-C13A, STREX-C23A, STREX-C25A compared to the STREX control (at +40 mV 0.13 ± 0.01, 0.1 ± 0.01, 0.11 ± 0.16, 0.32 ± 0.03 respectively, with $p<0.01$ ANOVA plus Tukey posthoc). This suggested that mutation of these residues affected the absolute channel activity. Surprisingly, under these conditions the C12A mutant did not have a significantly different starting Po compared to STREX despite being a significant contributor to the STREX variants enhanced response to calcium influx as seen in the FMP assays (Section 3.2.3).

The effects of acute hypoxia on the mutant channels, in isolated inside-out patches, was investigated in parallel with the effect of hypoxia on wildtype STREX channels, on the same batch of transfected HEK293 cells to avoid any batch to batch variation of cellular responses.

The STREX-C12A mutant showed a similar response to hypoxia as for STREX channels (Figure 4.3), upon perfusion with hypoxic solution at the intracellular face of the patch (as described in Section 2.4.5) the STREX-C12A responded to a decrease in oxygen tension by a decrease in channel activity which was reversible upon washout with normoxic solution (mean % inhibition was 67 ± 6 % , n = 3, $p<0.001$ Kruskal-Wallis test with Dunns posthoc compared to respective normoxic control, Figure 4.10). Although STREX-C13A had a reduced starting Po compared to STREX, the mutation did not affect the ability of the channel to respond to hypoxia with a decrease in channel activity (Figure 4.4 & 4.10, observed mean % inhibition was 41 ± 15 % , n = 3, $p<0.001$ Kruskal-Wallis test with Dunns posthoc compared to respective normoxic control). In a similar fashion the STREX-C16A mutant also responded to changes in oxygen tension (Figure 4.5), although the decrease in channel activity induced by

Figure 4.3
Effect of hypoxia on STREX-C12A channels

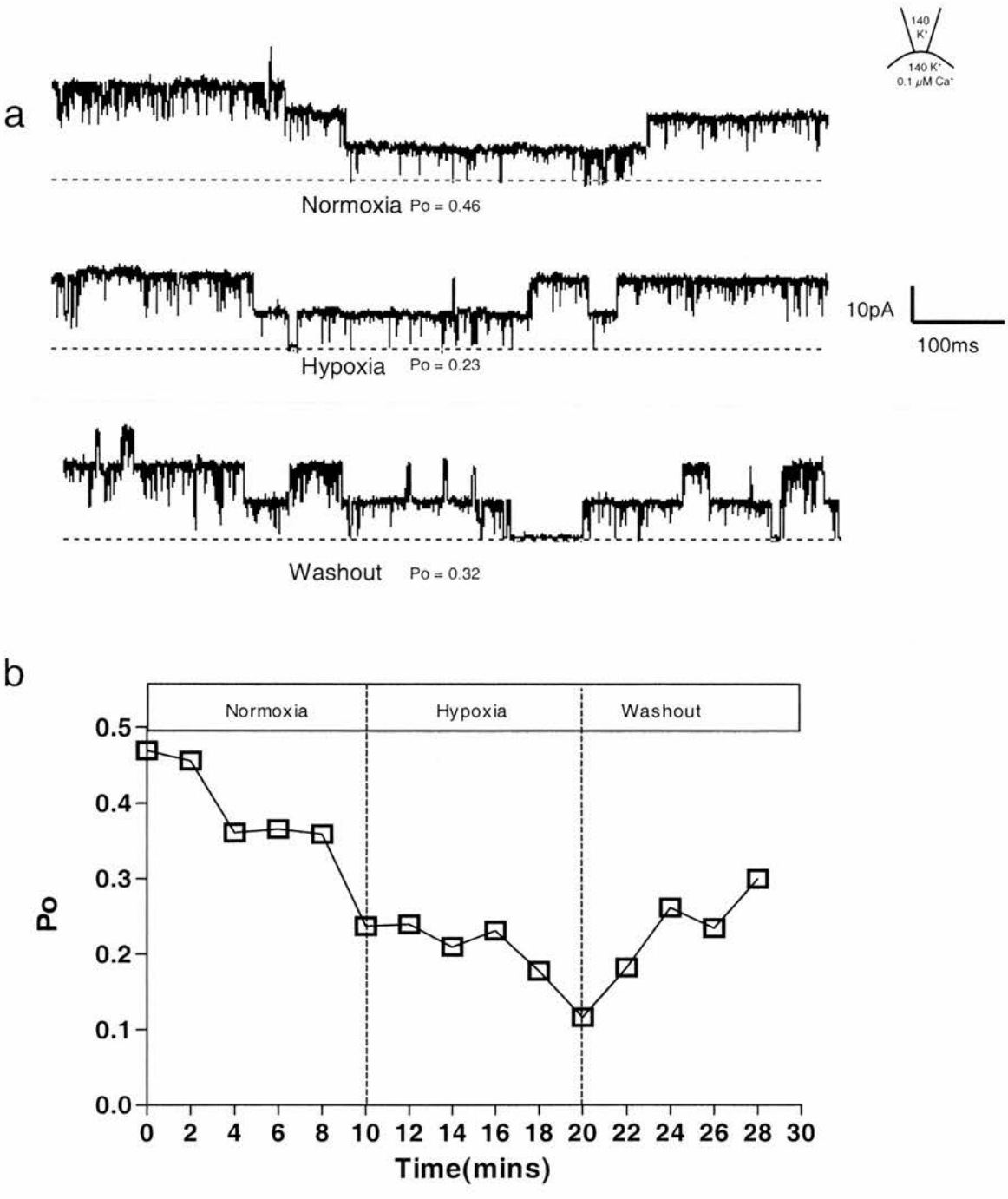


Figure 4.3 Example recordings of single channel activity in an excised inside-out patch, of STREX-C12A channels transiently expressed in HEK293 cells at 40mV. Panel a shows recordings under conditions of normoxia, hypoxia and then washout, Po values are indicated. Panel b illustrates the response over the time course of the example experiment.

Figure 4.4
Effect of hypoxia on STREX-C13A channels

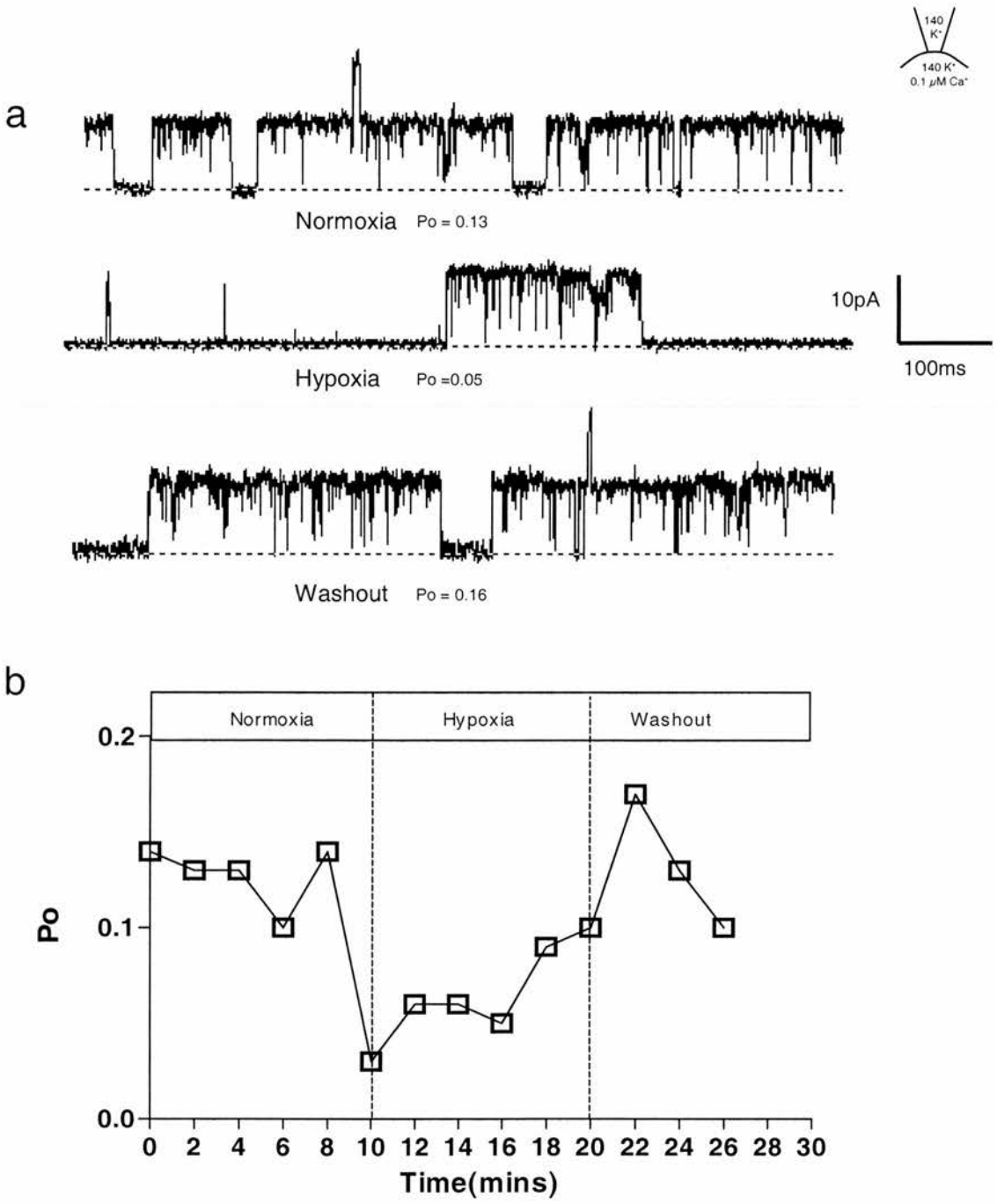


Figure 4.4 Example recordings of single channel activity in an excised inside-out patch, of STREX-C13A channels transiently expressed in HEK293 cells at 40mV. Panel a shows recordings under conditions of normoxia, hypoxia and then washout, Po values are indicated. Panel b illustrates the response over the time course of the example experiment.

(Data courtesy I.C.M. Rowe)

Figure 4.5
Effect of hypoxia on STREX-C16A channels

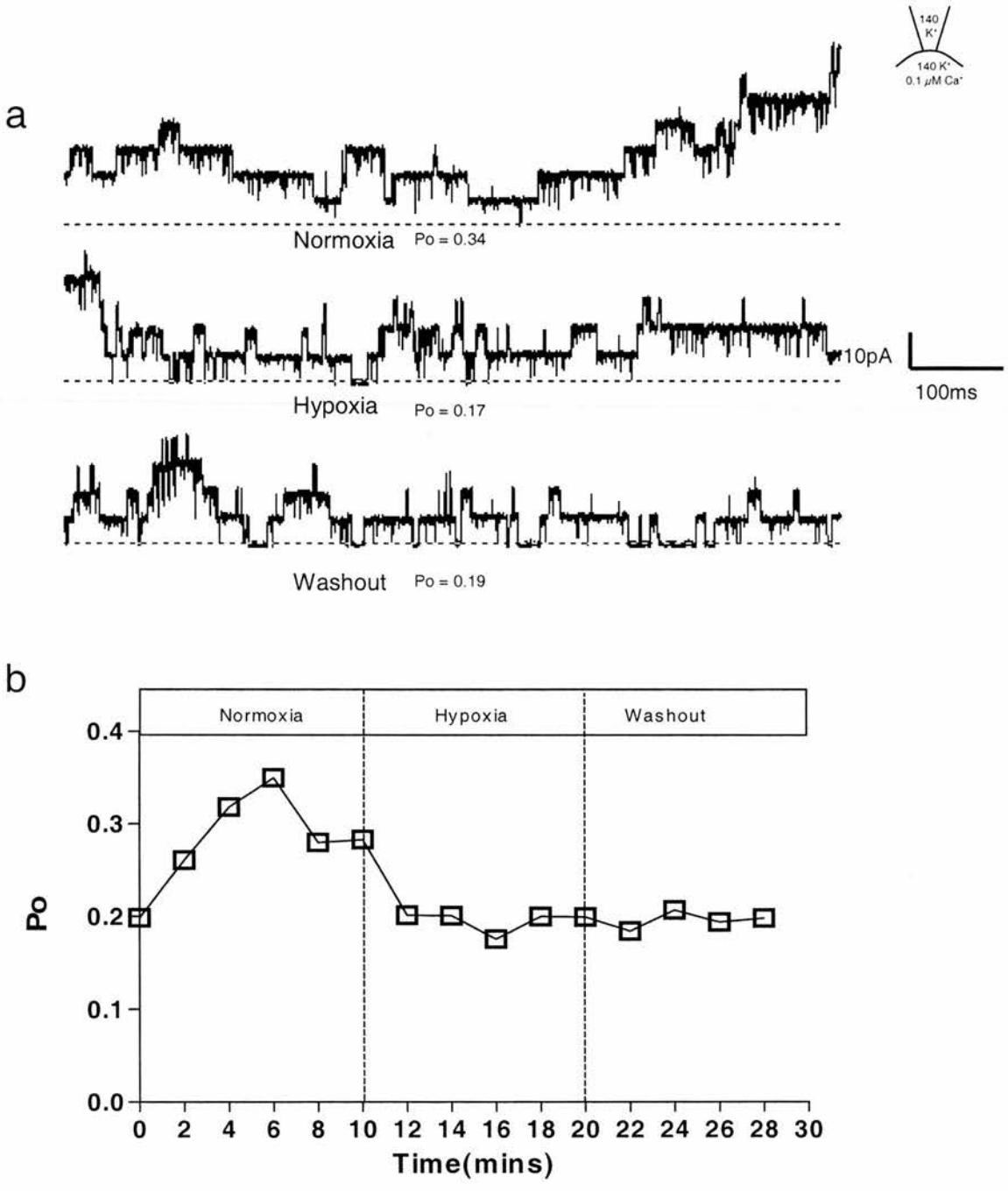


Figure 4.5 Example recordings of single channel activity in an excised inside-out patch, of STREX-C16A channels transiently expressed in HEK293 cells at 40mV. Panel a shows control recordings in normoxic and hypoxic solution with the washout response, Po values are indicated. Panel b illustrates the example time course response.

hypoxia was not always reversible (mean % inhibition was 56 ± 14 % , n = 3, $p<0.001$ Kruskal-Wallis test with Dunns posthoc compared to respective normoxic control, Figure 4.10).

In agreement with previously published data (McCartney *et al.*, 2005) the STREX-C23:25A mutant did not respond to hypoxia with a decrease in channel activity (8 ± 5.7 % increase in channel activity observed under hypoxic conditions compared to normoxic control, n = 3, Figure 4.6 & 4.10). Individual point mutants of the cysteine residues were constructed in an attempt to elucidate if either cysteine residue alone was responsible for the sensitivity to hypoxia.

The STREX-C23A mutant had a significantly reduced channel activity in comparison to STREX under conditions of normoxia. The STREX-C23A mutant failed to respond to changes in oxygen tension at the intracellular face of the patch (Figure 4.7, 2 ±8% increase in channel activity under hypoxic conditions compared to normoxic control, n = 5). Therefore revealing that this cysteine residue is critical for the STREX hypoxic response (Figure 4.10).

Figure 4.6
Effect of hypoxia on STREX-C23:25A channels

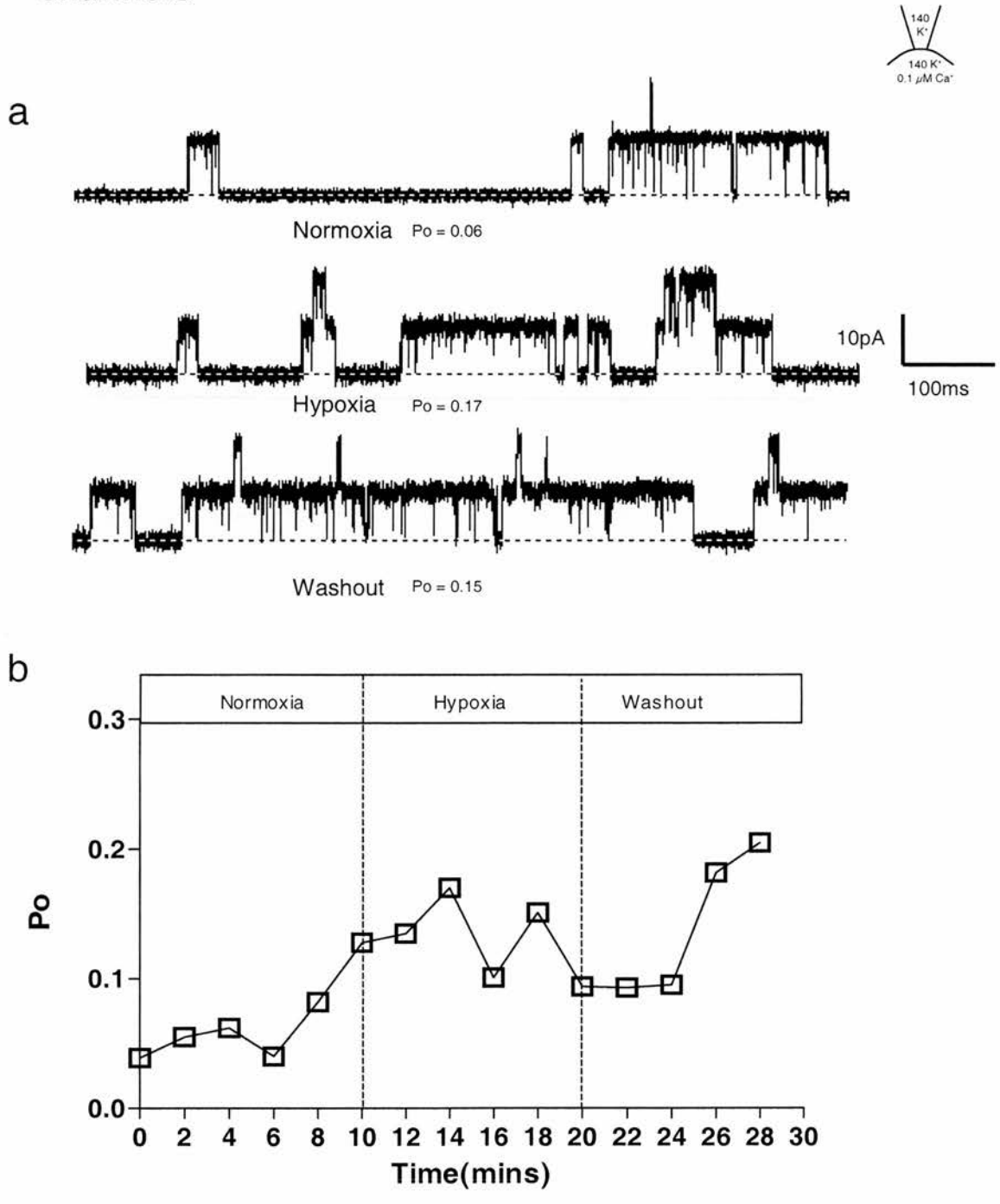


Figure 4.6 Example recordings of single channel activity in an excised inside-out patch, of STREX-C23:25A channels transiently expressed in HEK293 cells at 40mV. Panel a shows control recordings in normoxic and hypoxic solution with the washout response, Po values are indicated. Panel b illustrates the example time course response.

Figure 4.7
Effect of hypoxia on STREX-C23A channels

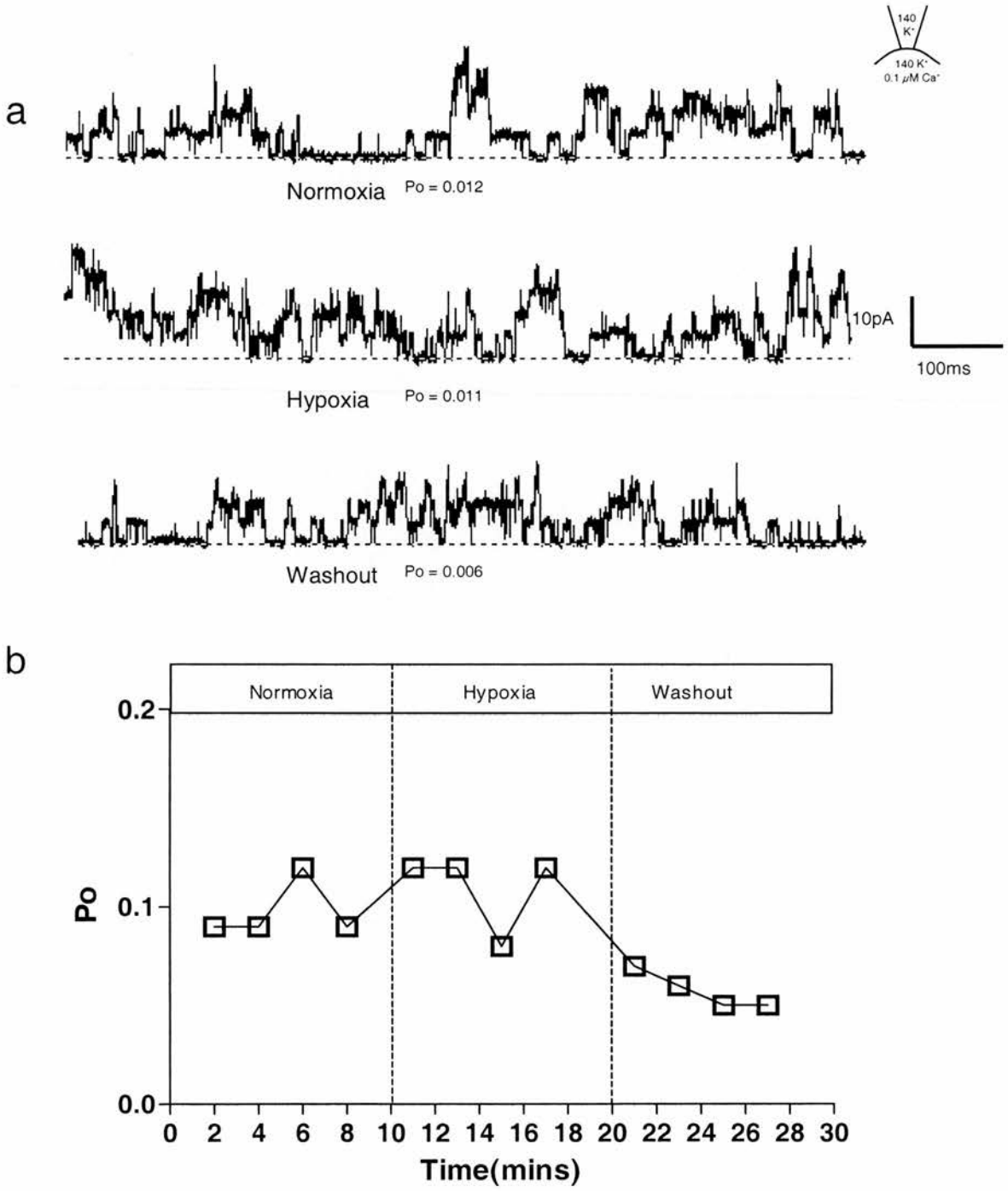


Figure 4.7 Example recordings of single channel activity in an excised inside-out patch, of STREX-C23A channels transiently expressed in HEK293 cells at 20mV. Panel a shows control recordings in normoxic solution, and hypoxic solution with the washout response, Po values are indicated. Panel b illustrates the example time course response.

(Data courtesy I.C.M. Rowe)

Similar to the STREX-C23A channel activity, the STREX-C25A baseline activity was significantly reduced compared to STREX. The STREX-C25A mutant had a significantly reduced channel activity in comparison to STREX under conditions of normoxia, nonetheless perfusion of hypoxic solution to the intracellular face of the patch caused a significant decrease in channel activity (Figure 4.8) that was reversible upon washout with normoxic solution (mean % inhibition was 51 ±30% , n = 6, $p<0.01$ Kruskal-Wallis test with Dunns posthoc compared to respective normoxic control, Figure 4.10) thus implying that the C25 residue is not crucial for the STREX hypoxic response.

The STREX-C51 residue is adjacent to a serine residue S52 and could potentially have similar properties to the CSC motif (Figure 4.1). However mutation of this residue to alanine did not prevent hypoxic inhibition of the channel (Figure 4.9 & 4.10, observed mean % inhibition was 47 ±5%, n = 5, $p<0.001$ Kruskal-Wallis test with Dunns posthoc compared to respective normoxic control).

Figure 4.8
Effect of hypoxia on STREX-C25A channels

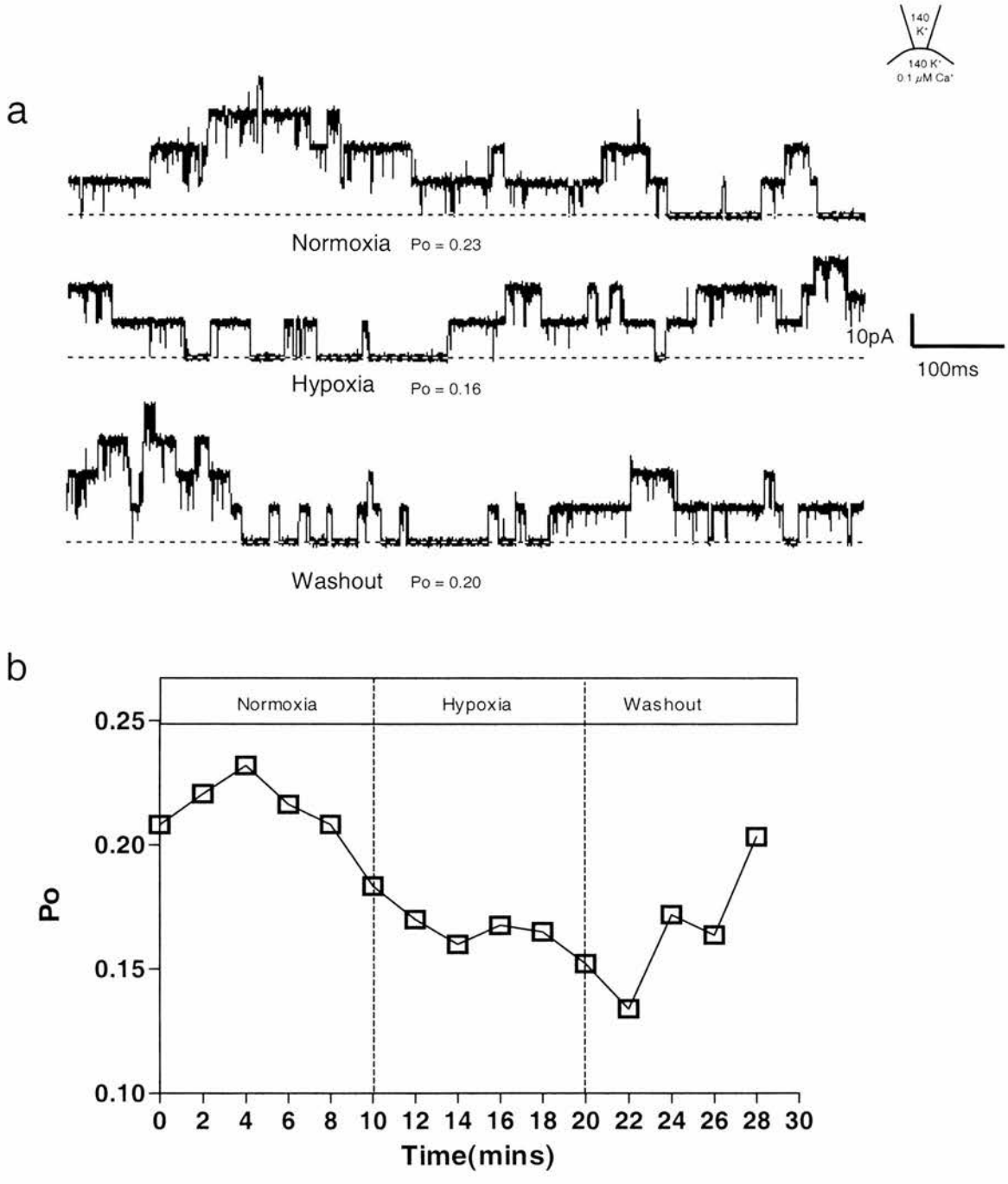


Figure 4.8 Example recordings of single channel activity in an excised inside-out patch, of STREX-C25A channels transiently expressed in HEK293 cells at 40mV. Panel a shows control recordings in normoxic and hypoxic solution with the washout response, Po values are indicated. Panel b illustrates the example time course response.

Figure 4.9
Effect of hypoxia on STREX-C51A channels

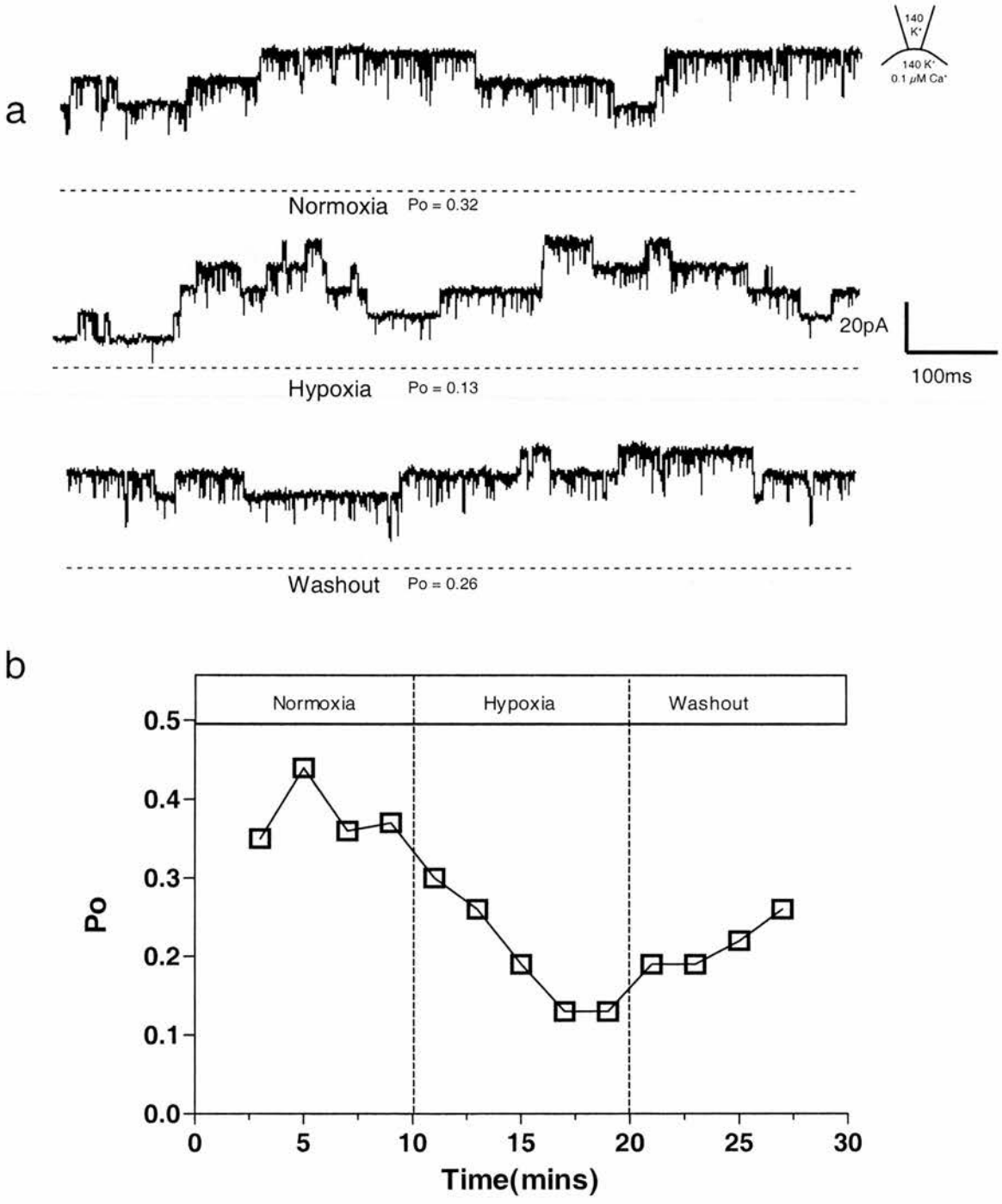


Figure 4.9 Example recordings of single channel activity in an excised inside-out patch, of STREX-C51A channels transiently expressed in HEK293 cells at 60mV. Panel a shows control recordings in normoxic and hypoxic solution with the washout response, Po values are indicated. Panel b illustrates the example time course response.

(Data courtesy I.C.M. Rowe)

Figure 4.10
Effect of hypoxia on all STREX cysteine mutant channels

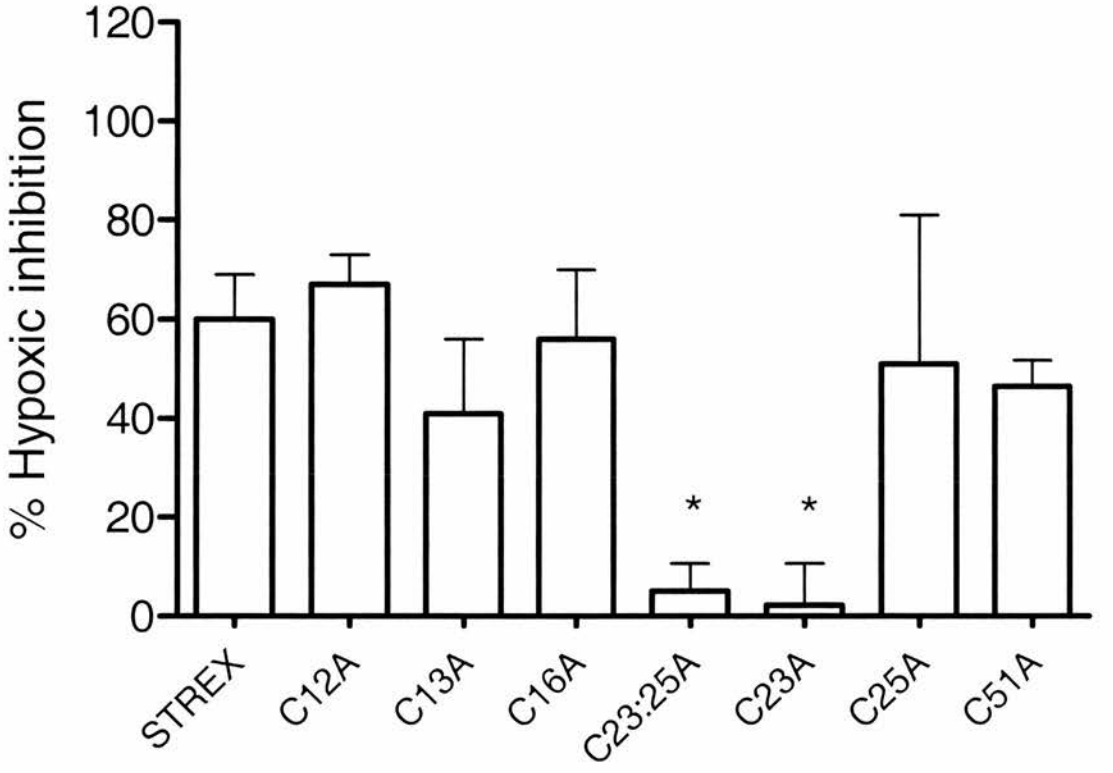


Figure 4.10 The summary bar graph above illustrates the % change in channel activity of STREX and the cysteine mutant channels in those patches that demonstrated inhibition under conditions of hypoxia when compared to their respective normoxic controls. * $p<0.05$ Kruskal-Wallis test with Dunns posthoc compared to wildtype STREX hypoxia response.

4.3 Summary

Acute hypoxia inhibits the activity of STREX BK channels, previously this inhibition was attributed to the -CSC- motif present in the STREX insert region (McCartney *et al.*, 2005). Consequently it was proposed that hypoxia may induce a conformational change in the channel structure possibly via disruption of interactions mediated by the cysteine residues within the -CSC- motif. Cysteine-cysteine interactions in other proteins are also sensitive to REDOX modifications (Park *et al.*, 1999; Graumann *et al.*, 2001; Kurek *et al.*, 2002; Ghezzi & Bonetto, 2003) thus all cysteine residues in STREX were investigated to determine any potential role they may have in hypoxia sensing.

Site directed mutagenesis and electrophysiology experiments here revealed that only the C23 residue (of the previously proposed hypoxia sensitive -CSC- motif) is the crucial cysteine residue responsible for the STREX hypoxic response, as mutation of C25 (within the -CSC- motif) to alanine did not alter the channel's response to hypoxia. Similarly the C51 amino acid located next to a serine residue (S52) was expected to have similar properties to the -CSC- motif, however mutation of this site also did not alter the STREX hypoxic response.

The proposed mechanism of action was that the cysteine residues within STREX form disulphide bonds and hypoxia induced disruption of these bonds causes a subsequent change in channel activity. However from the data presented here we can conclude the partner cysteine of the C23 disulphide bond can not be within the STREX insert itself as mutation of the other STREX cysteine residues did not abolish the hypoxic response. Thus, it is possible that the C23 residue could form disulphide bonds with other cysteine residues within the same α subunit or indeed inter-subunit linkage could occur.

It is unlikely that C23 alone mediates the conformational changes necessary for the STREX hypoxic response, as mutation of the serine (S24) residue adjacent to C23 to alanine was shown to prevent hypoxic inhibition (McCartney *et al.*, 2005) without affecting channel baseline activity. The serine residue has a negatively charged nitrogen group which allows nucleophilic displacement of the reactive pair of lone electrons in the thiol group of the cysteine amino acid so promoting disulphide bond formation. Thereby the serine residue modifies the local environment of the hypoxia sensing motif to facilitate the rapid and reversible effects of reduced oxygen tension on STREX, and was investigated further in Chapter 5. So in fact the intrinsic hypoxia sensing ability of STREX may be

conferred by the evolutionary conserved cysteine-serine ($C_{23}S_{24}$) and not the previously proposed -CSC- motif.

So how does the C23 amino acid residue mediate the STREX hypoxia sensitivity? The most logical model would be via interactions with vicinal residues such as the S24 which could perhaps underlie a conformational change in the channel in response to hypoxia. Cysteine residues are responsible for forming disulphide bonds thus mediating a stabilising effect on protein structure. Thiol modifications however can occur selectively on reactive cysteine residues that are accessible on protein surfaces thus altering the protein function. So it could be that by mutating the C23 residue in STREX we are preventing not only the formation of disulphide bonds but also other thiol modifications particularly those that are mediated on C-terminus cysteines such as palmitoylation (Dunphy & Linder, 1998) and prenylation (Pechlivanis & Kuhlmann, 2006). It could be that these modifications play a key role in the structural changes which hypoxia induces in STREX.

BK channels are tetramers of four pore-forming α subunits. It is wholly possible that the single mutation of the C23 residue prevents intra-subunit interactions such as disulphide bond formation between the different α subunits, as well as inter-subunit interactions along the C-terminus of the channel. TEA insensitive subunits co-expressed with wildtype TEA sensitive subunits have been used to determine that channel stoichiometry of BK alters the phosphorylation sensitivities of the channel (Tian *et al.*, 2004). A similar approach could therefore be used to investigate if inter-subunit interactions have a role in the STREX hypoxic response. Channel homomers and heteromers of the STREX-C23A mutant and STREX wildtype channel could be constructed to determine if inter-subunit interactions mediated by the C23 residue are altered during hypoxic exposure and could be responsible for the change in BK channel activity under conditions of low oxygen tension. Although technically very demanding co-expression studies in which the stoichiometry ratio of STREX wildtype and mutants was controlled using the TEA-insensitive α subunits could provide information on how many functional STREX α subunits are required for hypoxia sensitivity of the channel. Additionally the ratio of STREX subunits required for the hypoxia sensitivity using TEA insensitive ZERO α subunits could be investigated. This would be a vital step in understanding the

functional importance of STREX dependent hypoxia sensing in native cells that express more than one splice variant.

The lack of any crystallisation of STREX at present to elucidate the 3D structure means that to investigate if the STREX hypoxia response is mediated by a change in the functional conformation of the channel it would be necessary to employ novel fluorescence lifetime imaging microscopy (FLIM)-based FRET assays. FLIM can be used as an accurate and quantitative assay to study conformational changes of proteins in cells (Duncan *et al.*, 2004), so if fluorescent fusion proteins were tagged onto the functional STREX channel or indeed truncated C-terminus segments this would shed light on the structural changes that occur in STREX during exposure to hypoxic conditions.

The reduced calcium sensitivity and baseline channel activity of the C23 and C25 mutants has been demonstrated here and in previous electrophysiology assays (McCartney *et al.*, 2005; Saleem *et al.*, 2007). However the FMP assay (Section 3.2.3) illustrated that although all cysteine residues altered the ionomycin-induced hyperpolarisation, only the C12 and C13 residues

significantly reduced ionomycin-induced hyperpolarisation thus affecting the ability of the channel to respond to calcium influx. Interestingly though the C13 residue but not the C12 residues had significantly reduced baseline activity in the electrophysiology experiments. This difference highlights the different stimulus used in both assays; the FMP assay measures the calcium driven response of the BK channels whereas in electrophysiology the changes in channel activity measured are driven both via calcium and voltage difference. Perhaps the C12 and C13 residues alter calcium sensitivity of the channel whereas the C23 and C25 residues are affected by the calcium and voltage sensitivity of the channel. To distinguish between these two it would be useful to conduct electrophysiology experiments in the absence of calcium thereby looking at the voltage driven response of the cysteine mutants thus elucidating the role these four cysteine residues play in the apparent calcium and voltage sensitivity of STREX.

In conclusion, these studies identified C23 as the only critical residue within STREX responsible for the STREX hypoxic response. Hypoxia may disrupt vital interactions that this cysteine residue may mediate with other parts of the channel structure, perhaps by modulation of intra or inter-subunit disulphide

bonds. However we can not eliminate disulphide independent reactions which may contribute to the decrease in channel activity observed in STREX under conditions of low oxygen tension.

Chapter Five: Potential role of ATP and lithium in controlling the sensitivity of STREX channels to hypoxia

5.1 Introduction

5.1.1 Identification and role of phosphorylation motif in STREX

BK channels are known mediators of the body's response to hypoxia. They contribute to the oxygen sensing role of specific tissues, such as the carotid body and neuroepithelia (Riesco-Fagundo *et al.*, 2001; Jovanovic *et al.*, 2003) and in determining cellular excitability in smooth muscles and neurones (Liu *et al.*, 1999; Porter *et al.*, 2001). As discussed in Section 1.5 and 4.1 several mechanisms have been proposed for the hypoxia-mediated regulation of BK channels, including changes in redox state (Tang *et al.*, 2004), activation of AMP-kinase (Wyatt *et al.*, 2007) and heme-oxygenase dependent carbon monoxide (CO) production (Williams *et al.*, 2004).

Recent research in our lab has revealed a novel mode of hypoxia sensitivity conferred by the STREX splice variant of the BK channel, which has an evolutionary conserved cysteine rich insert. The STREX channel is rapidly inhibited by a decrease in oxygen tension that is calcium dependent and reversible (McCartney *et al.*, 2005). It has been established that the hypoxic response is abolished when increasing calcium concentration to 10 μM and that

other splice variants of the BK channel, ZERO and IYF, do not show any change in activity in low oxygen tension, so this response is limited to the STREX variant. The hypoxic inhibition has been shown to be distinct from previously described hypoxic responses, thus understanding this novel form of regulation is important in elucidating the physiological role of BK channels.

So how does hypoxia cause a decrease in STREX channel activity? A putative "hypoxia-sensing" motif has been identified within the STREX insert (-CSC-) that incorporates a serine residue (S24) flanked by cysteine residues (C23; C25) (McCartney *et al.*, 2005). This -CSC- motif lies within a RxxS motif (RxCS within the STREX insert) which is a putative serine-threonine kinase phosphorylation motif (Pearson & Kemp, 1991; Aitken, 1999; Tian *et al.*, 2001a; van Montfort *et al.*, 2003; Tian *et al.*, 2004), and can potentially be phosphorylated by PKG, PKC, PKA and CamKinase. It is possible that the response of STREX channels to hypoxia is an intrinsic BK channel effect i.e. a decrease in oxygen tension causes a structural change in the channel protein itself, or a secondary effect following changes in kinase behaviour or free radical production upon exposure to hypoxia, which then results in structural changes that influence channel activity.

Acute and chronic hypoxia alters ATP levels in mammalian cells (Saikumar *et al.*, 1998; Tang *et al.*, 2004; Turner *et al.*, 2004). A recent study showed that hypoxia prevents mitochondrial oxidative phosphorylation in the carotid body which leads to an increase in the AMP/ATP ratio (Wyatt *et al.*, 2007). This in turn activates the AMP kinase which inhibits BK channels and causes depolarisation of the carotid body. Therefore changes in ATP concentration during hypoxia could activate kinases that are closely associated with the channel protein on the cell membrane and alter the phosphorylation state of the channel. Thus, phosphorylation of channel protein could be mediating the STREX channels response to hypoxia, as previous data was obtained in the absence of ATP (McCartney *et al.*, 2005). However it could be that the STREX channel is required to be in a phosphorylated state to elicit this decrease in channel activity in response to hypoxia, or indeed that the decrease in oxygen tension causes phosphorylation of the STREX channel which induces the change in channel activity.

The strategy to investigate potential ATP modulation of the STREX channels response to hypoxia via the RxCS site was to use patch clamp electrophysiology to investigate the effect of hypoxia on excised inside-out patches. Firstly it was

investigated if the presence of ATP at the intracellular face of the channel could modulate the STREX channels response to low oxygen tension. The second strategy was to take a site-directed mutagenesis approach by mutating the RxCS motif, making phosphonull and phosphomimetic mutants (R21A, S24A and S24E) to test if phosphorylation of this motif is important for STREX channel sensitivity to hypoxia.

5.2 Results

5.2.1 Effects of ATP on the response of STREX channels to hypoxia

As shown in Section 4.2 acute exposure to hypoxia resulted in a reversible inhibition of STREX channel activity in isolated inside-out patches from transiently transfected HEK293 cells, in the absence of ATP or other exogenous intracellular metabolites under symmetrical potassium conditions at +40 mV and in the presence of 0.1 μM intracellular free calcium (Figure 5.1 & 5.4). Under these conditions STREX Po (open probability) during the normoxic stabilisation phase directly after excision of the patch was stable and did not vary significantly from patch-to-patch, with a range of 0.3 - 0.6. Upon perfusion of

the intracellular face of the patch with hypoxic solution (pO_2 < 20mm/Hg) a rapid and sustained significant decrease in Po was observed (mean % inhibition was 60 ± 9 % , n = 13, $p<0.001$ Kruskal-Wallis test with Dunns posthoc compared to respective normoxic control) that reversed upon washout with normoxic solution (Figure 5.1 & 5.4). Thus in the absence of ATP, hypoxia reduces STREX channel activity in all patches examined and closely follows the change in Po as the intracellular oxygen tension changes.

To address whether the presence of ATP at the intracellular face of the patch modified the response to hypoxia in STREX, channels were recorded under identical conditions except that the intracellular face of the patch was exposed to 1 mM ATP immediately upon patch excision and maintained throughout the experiment. The effect of hypoxia on STREX channels in isolated inside-out patches in the presence of ATP was performed in parallel with experiments in the absence of ATP, on the same batch of transfected HEK293 cells, to avoid any batch-to-batch variation of cellular responses. STREX Po was not significantly different in the presence and absence of ATP during the control normoxic period ranging from 0.19 - 0.68 (Figure 5. 2 & 5.3) and had no significant effect on single channel amplitude (at +40 mV unitary current amplitude was 10.0 ± 0.1

pA n = 13 in the absence of ATP and in the presence of ATP was 9.6 ± 0.1 pA n=15).

Interestingly, two contrasting phenotypes emerged in the presence of ATP. In 50% of the experiments there was robust inhibition in low oxygen tension (mean inhibition was 55 ± 12 %, n = 7, $p<0.001$ Kruskal-Wallis test with Dunns posthoc compared to respective normoxic control), identical to the response of STREX channels to hypoxia in the absence of ATP that again reversed upon washout into normoxic solution (Figure 5.2 & 5.4). Conversely under identical conditions, in the remaining 50% of patches in the presence of ATP, a significant activation of STREX channels was seen upon exposure to hypoxia (Figure 5.3). In these patches, there was a rapid and sustained increase in Po (mean activation was 50 ± 12 %, n = 7 $p<0.001$ Kruskal-Wallis test with Dunns posthoc compared to respective normoxic control) that reversed upon washout into normoxia solution (Figure 5.3 & 5.4).

Figure 5.1
Hypoxia inhibits STREX channels in the absence of ATP

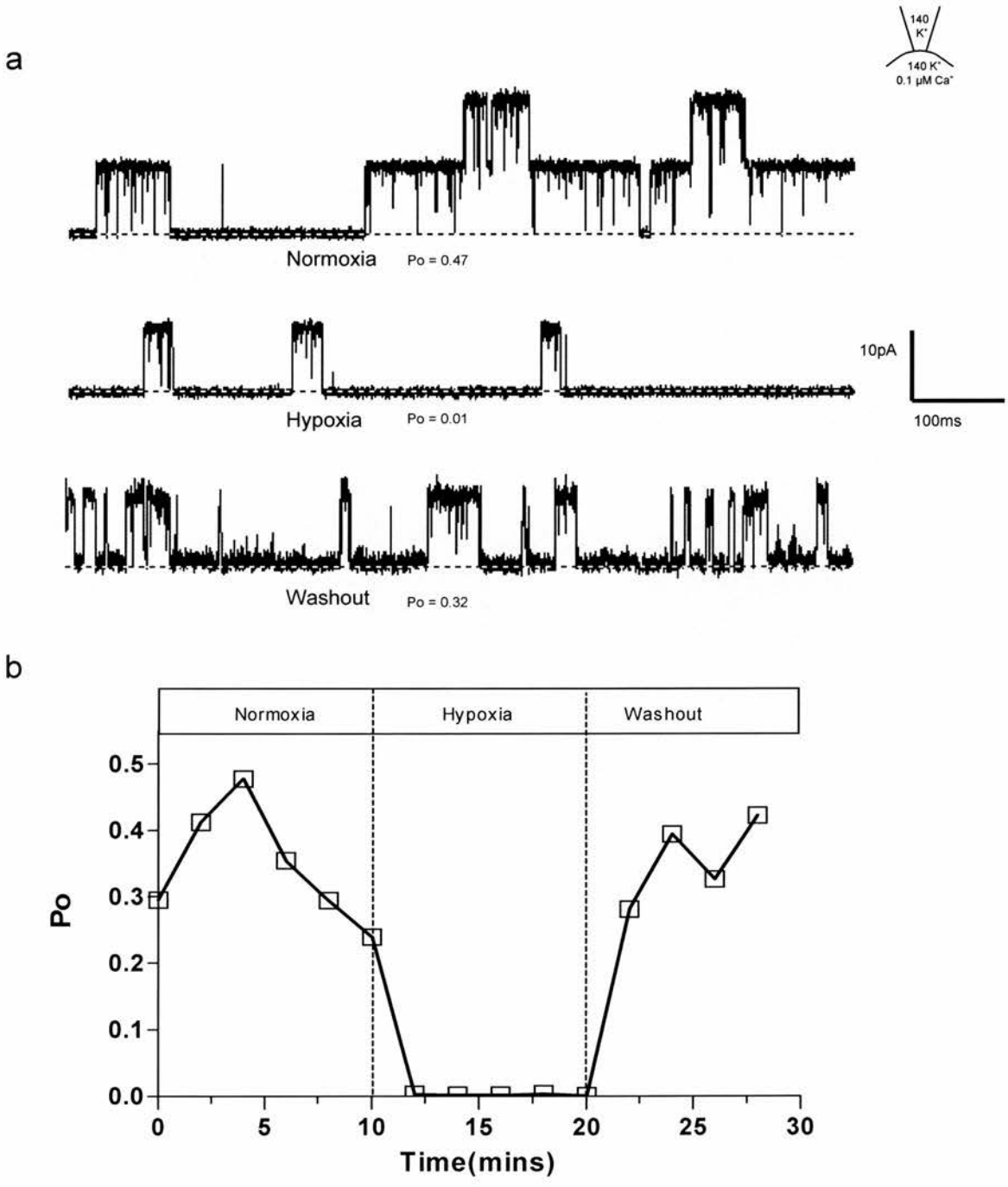


Figure 5.1 Example recordings of single channel activity in an excised inside-out patch, of STREX channels transiently expressed in HEK293 cells at +40mV. Panel a shows recordings in normoxic solution, hypoxia induced a significant decrease in channel activity as indicated by Po values, which was reversible during washout with normoxic solution. The example time course of the response is illustrated in panel b.

Figure 5.2
Effect of hypoxia on STREX channel activity in the presence of 1mM ATP: example of inhibition

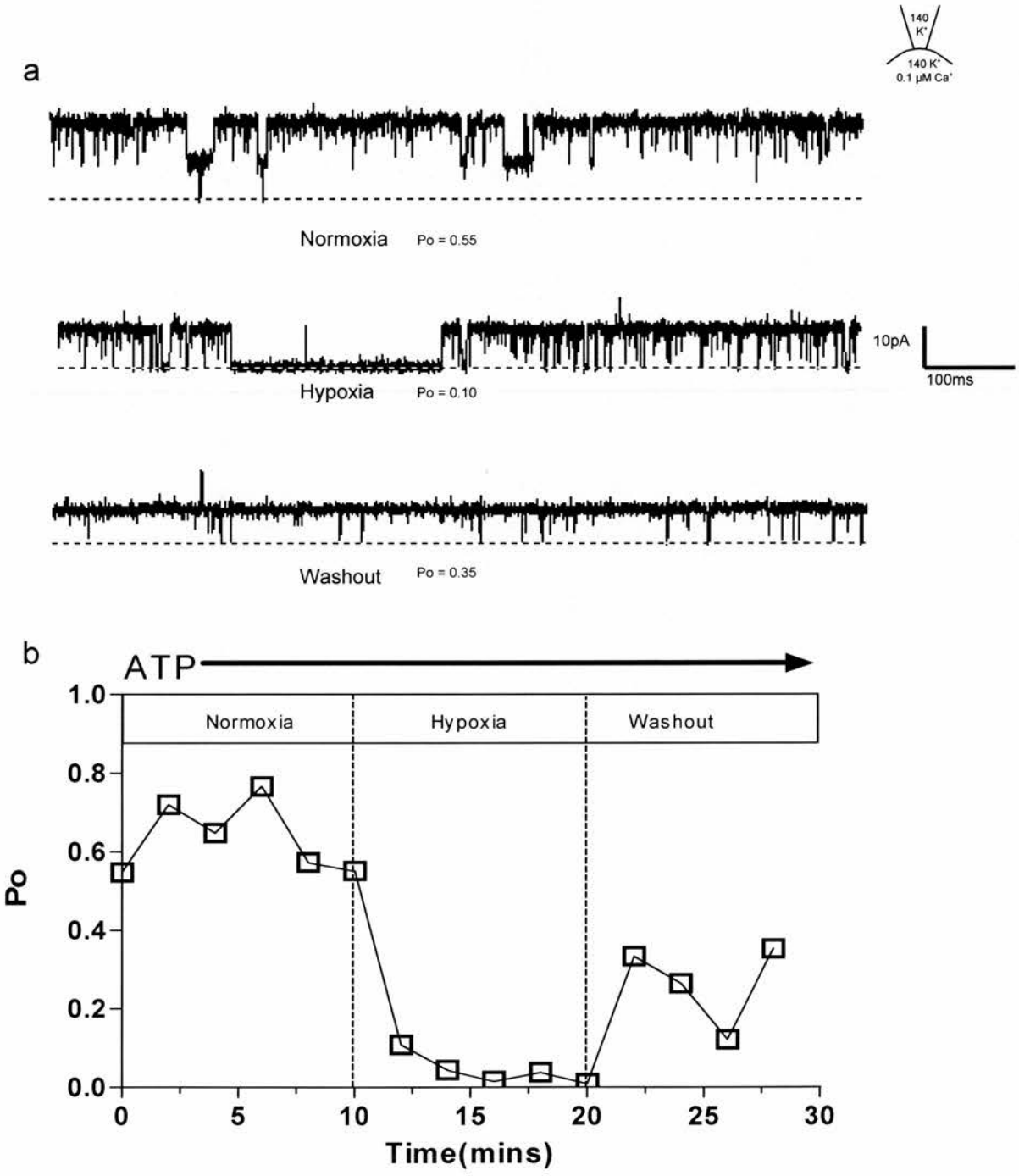


Figure 5.2 Example recordings of single channel activity in an excised inside-out patch, STREX channels expressed in HEK293 cells at +40mV in 1mM ATP. Panel a shows control recordings in normoxic solution, Po values are indicated. Hypoxia induced a significant reversible decrease in channel activity. The example time course of the response is illustrated in panel b.

Figure 5.3
Effect of hypoxia on STREX channels in the presence of 1mM ATP: example of activation

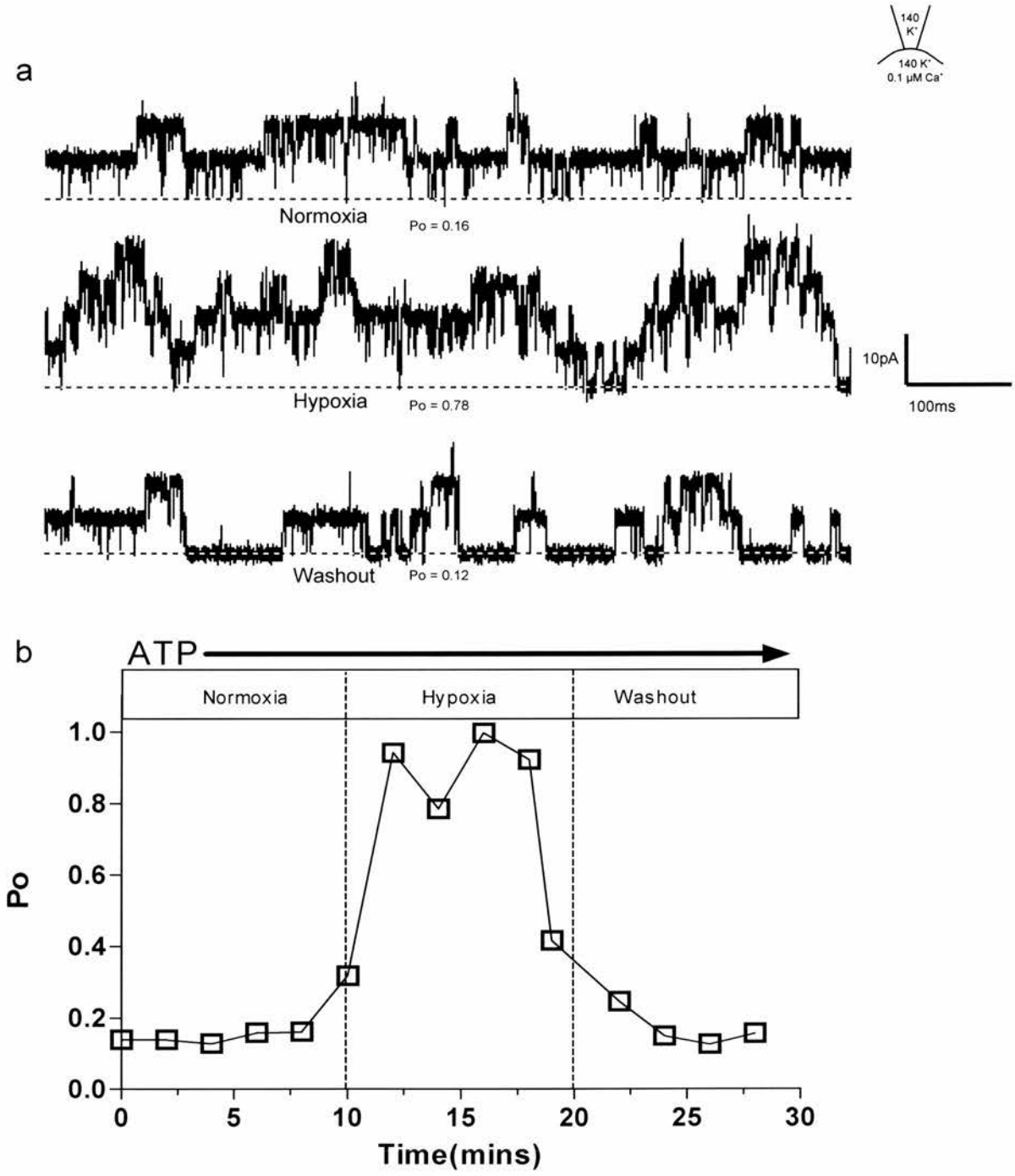


Figure 5.3 Example recordings of single channel activity in an excised inside-out patch, STREX channels expressed in HEK293 cells at +40mV in 1mM ATP. Panel a shows control recordings in normoxic solution, Po values are indicated. Hypoxia induced a significant increase in channel activity as indicated with the reversibility of the response seen upon washout with normoxic solution. The example time course of the response is illustrated in panel b.

Figure 5.4
Summary of STREX channels response to changes in oxygen tension in the presence and absence of 1mM ATP

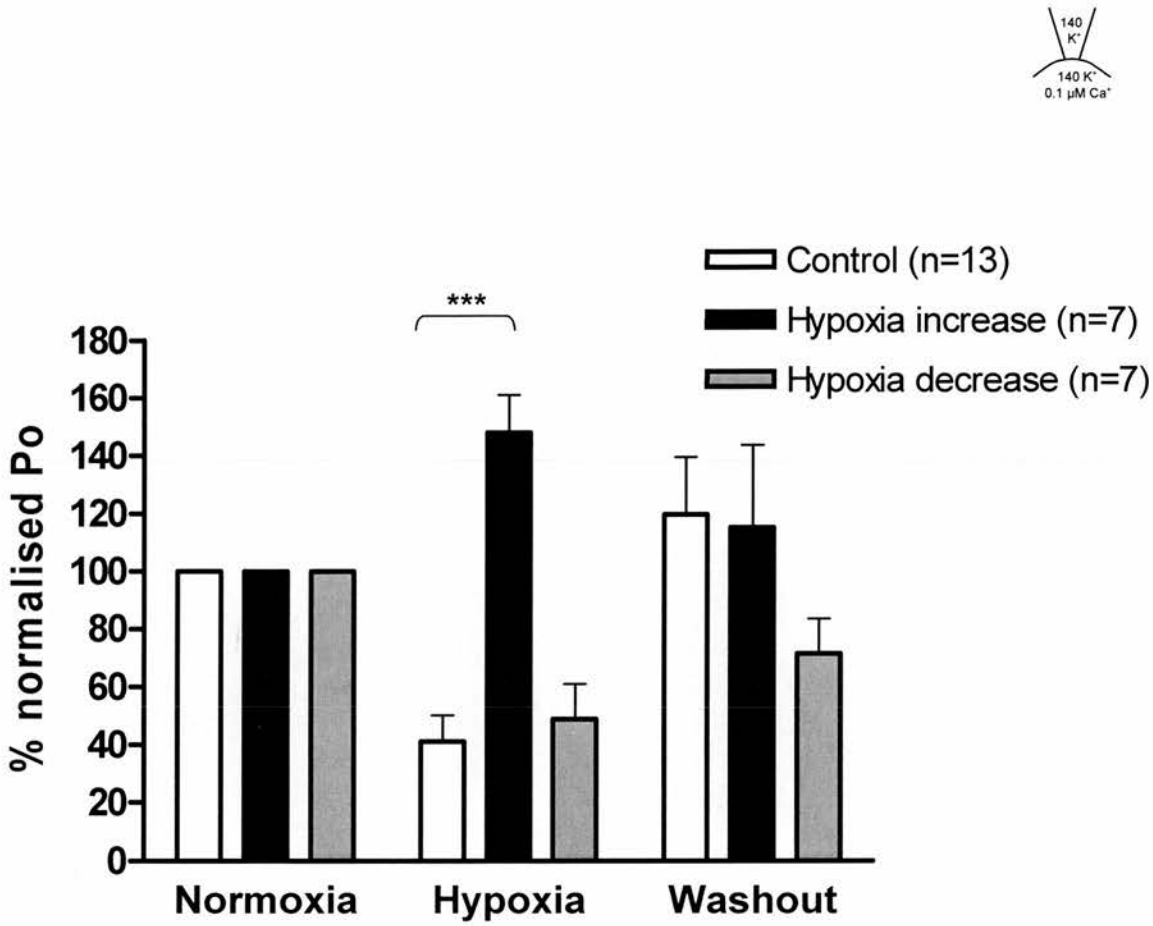


Figure 5.4 Summary graph showing the mean STREX responses to hypoxia with and without ATP as indicated. Two groups have emerged with the ATP treatments with half showing a decrease in Po activity and half displaying an increase. Data mean ± sem with n numbers indicated, *** $p<0.001$ Kruskal-Wallis test with Dunns posthoc compared to STREX channels control response to hypoxic conditions. (Hypoxia increase = increase in STREX channel activity under hypoxic conditions in presence of 1mM ATP, Hypoxia decrease = decrease in STREX channel activity under identical hypoxic conditions in the presence of 1mM ATP)

As the effect of exposing inside out patches to ATP resulted in two distinct populations of response, inhibition and activation respectively, these data would suggest that ATP does not exert a direct effect on the channel per se. Rather, it suggests that the effect of ATP is mediated via a component that is present in only 50% of patches and that may be lost upon patch excision in some patches. For example, in the patches in which inhibition was observed the ATP-sensitive cellular factor may be absent thus the response to hypoxia is identical to that observed in the absence of ATP. Conversely, in patches where the ATP-sensitive factor remains associated, ATP switches the hypoxia response to activation. However, these data do not reveal whether the effect of ATP is mediated via a phosphorylation (or ATP-hydrolysis dependent) reaction or if the effect of ATP is via direct ATP binding.

5.2.2 Mutating the putative RxCS phosphorylation motif abolishes the response of STREX channels to hypoxia

Section 4 describes the investigation of the hypoxia sensing motif within the STREX channel known as the -CSC- motif (McCartney *et al.*, 2005). This motif has been identified as a putative serine/threonine kinase consensus

phosphorylation motif, RxxS (Figure 5.5, RxCS in STREX) (Pearson & Kemp, 1991; Aitken, 1999) and therefore could undergo ATP-dependent phosphorylation, hence modifying the response to hypoxia.

To resolve the role of phosphorylation on the response of STREX channels to hypoxia, it would be necessary to look at the potential pharmacology involved perhaps utilising broad spectrum phosphatase or kinase inhibitors. However, as two different responses of STREX channels to hypoxia are observed in the presence of ATP it would be difficult to prove the effect of any kinase inhibitor i.e. the lack of ATP activation of STREX under hypoxia could be due to the absence of the ATP-sensitive factor in the excised patch rather than the effect of the kinase inhibitor blocking phosphorylation. Thus the number of pharmacology patch clamp electrophysiology experiments needed would be large and could potentially produce further complications as many of the inhibitors available are not specific enough and known to hit multiple targets.

Figure 5.5
Identification of a potential phosphorylation motif within the STREX insert

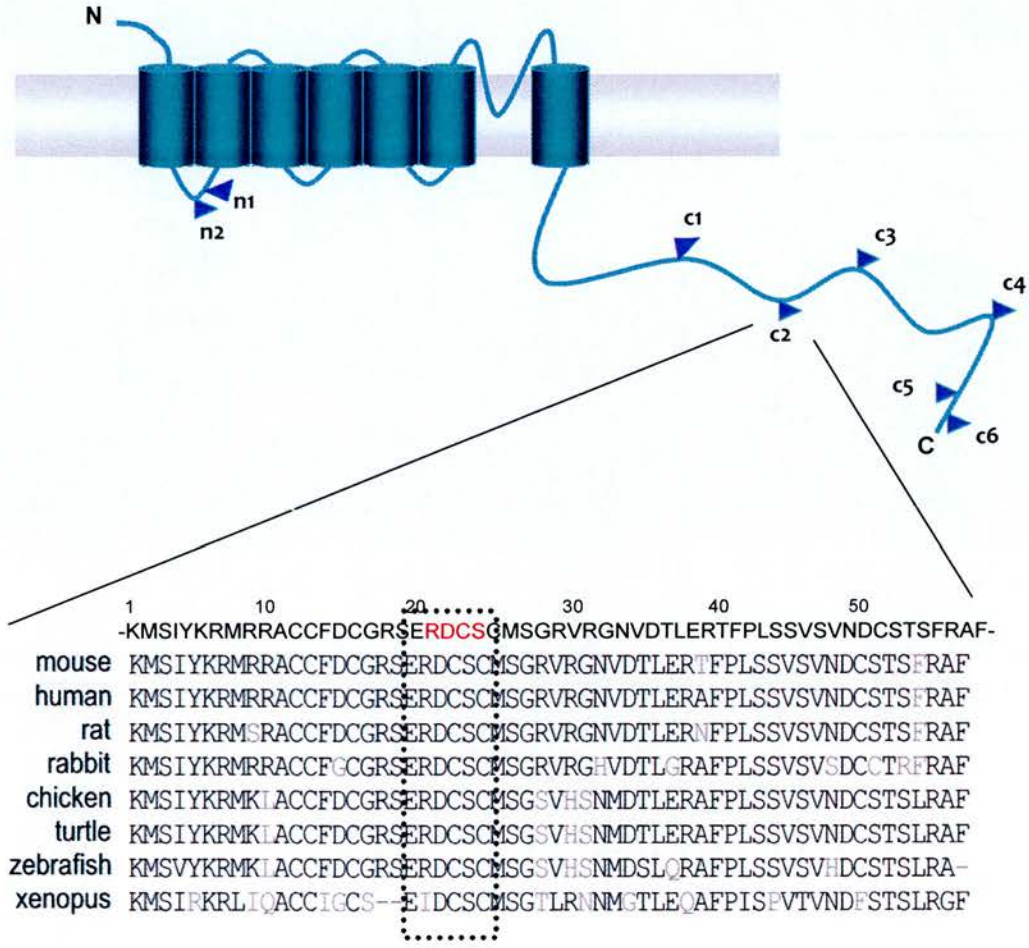


Figure 5.5 Evolutionary conserved RxCs motif within STREX

Consequently, I used a direct approach to elucidate the potential role of phosphorylation at the RxCS site in the response of STREX channels to hypoxia; this was investigated by using site-directed mutagenesis to mutate the RxCS site. This strategy would reveal the significance of abolishing the S24 phosphorylation site and the modulatory R21 site present in STREX, hence preventing any modulation on the residues by any intrinsic phosphorylation under hypoxic conditions. The mutation of S24 to glutamic acid (E), (a serine phosphomimetic amino acid), would also highlight the effect of a permanently phosphorylated site on the STREX inhibitory hypoxic response i.e. would activation be seen under hypoxic conditions as with the STREX ATP hypoxia activation response. I decided to engineer by site directed mutagenesis phosphonull and phosphomimetic mutants of S24 (S24A and S24E respectively) to test the significance of the RxCS phosphorylation site, on the STREX inhibitory hypoxic response. The R21 residue was also mutated to an alanine residue as this residue would be predicted to be critical for phosphorylation of the downstream serine residue by a protein kinase.

The mutations produced functional channels that were efficiently expressed in HEK293 cells. These mutants had no significant effect on single channel

amplitude as parallel experiments for wildtype STREX channels produced a unitary current amplitude of 9.5 ± 0.3 pA at +40mV with unitary current amplitude for the mutants being 9.5 ± 0.1 pA, 9.2 ± 0.1 pA, 9.4 ± 0.1 pA respectively for STREX-R21A, STREX-S24A and STREX-S24E (n = 5). The effects of acute hypoxia on the mutant channels in isolated inside-out patches in the absence of ATP were performed in parallel with wildtype STREX channel controls, on the same batch of transfected HEK293 cells to avoid any batch variation of cellular responses. There were no significant effects on the starting Po during the control normoxic period straight after excision of the patch between the mutants in comparison to the STREX controls conducted in parallel, with a range of 0.2 - 0.4 (Figure 5. 6, 5.7 & 5.8).

The phosphomimetic STREX-S24E mutant did not show any consistent changes in channel activity in response to perfusion with hypoxic solution (Figure 5.6 and 5.9), so this supposed "constitutively phosphorylated" state did not result in activation of STREX channels under conditions of low oxygen tension. Hypoxia did not have any significant effect on channel Po in the phosphonull mutant STREX-S24A (Figure 5.7 and 5.9); in fact the mutation at the S24 site caused Po to run-up during the experiment which did not correspond to

changes in oxygen tension. The STREX-R21A mutant showed a similar response (Figure 5.8 and 5.9), in that it did not exhibit any significant changes in channel activity in response to hypoxia but displayed a run up in channel activity that was quite variable. The summary bar chart (Figure 5.9) shows the variability in the responses of the mutants to changes in oxygen tension. Under identical conditions a robust inhibition of wild-type STREX channels was observed (57 ± 10 % mean inhibition of channel activity, $p<0.001$ Kruskal-Wallis $n= 5$). The difference in the responses between the mutants and the wildtype STREX channels can not be attributed to any differences in absolute Po or channel expression as there were no significant changes in both, in comparison to wildtype STREX channels.

The data elucidates that the intact RxCS site in STREX channels is necessary for the decrease in channel activity seen under conditions of low oxygen tension, as mutation of the key R and S amino acid residues in the motif to alanine, abolishes the inhibition in response to hypoxia. Therefore implying that response of STREX channels to hypoxia must be dependent on phosphorylation of the serine residue. Conversely though the phosphomimetic mutant STREX-S24E also, did not show any significant changes in channel behaviour under

hypoxia, suggesting that in fact phosphorylation of this residue does not play a role in the STREX channels hypoxic response. Thus as all three mutants did not respond to hypoxia it would seem that the absence of the channel regulation by hypoxia could be via a structural change and not phosphorylation per se. This is assuming that the STREX-S24E mutant is in fact behaving as a good phosphomimetic, as phosphomimetics are not always effective (Darman *et al.*, 2001; Darman & Forbush, 2002; Flemmer *et al.*, 2002), therefore it is reasonable not to completely eliminate the possible role of direct phosphorylation of the BK channel in hypoxic modulation in the presence of ATP.

In the presence of ATP two different phenotypes were observed indicating that maybe it is the inconsistent phosphorylation of the channel or a closely associated protein that is mediating this variable effect. Mutations of the phosphorylation motif however indicate that it would be unlikely that direct phosphorylation of the channel at the RxCS site, by a closely associated kinase, is involved in modulating the hypoxic response; rather a structural change within the channel could contribute to the ATP-dependent modulation of the STREX hypoxic response.

Figure 5.6
Hypoxia does not cause any significant changes in Po of STREX:S24E

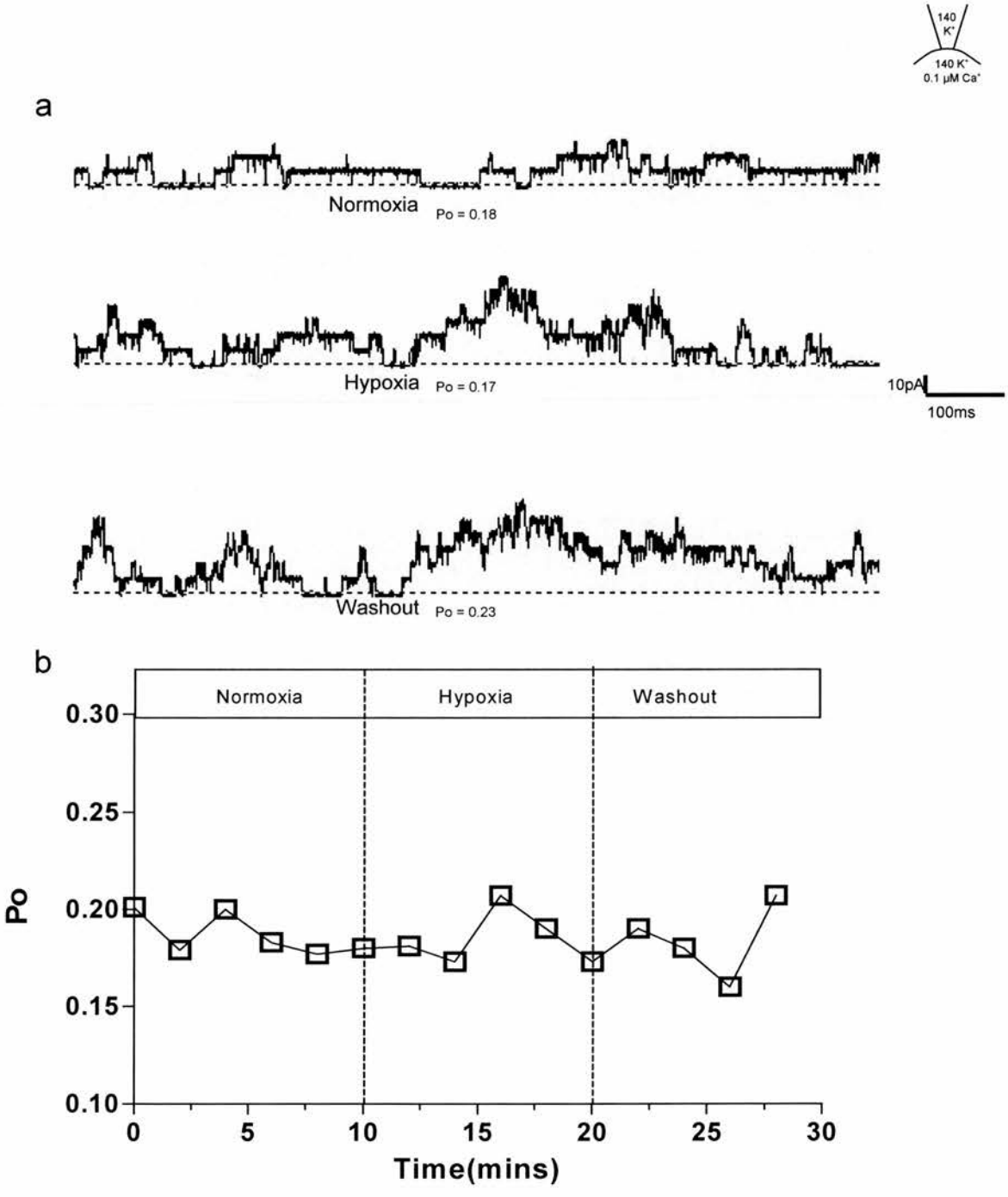


Figure 5.6 Example recordings of single channel activity in an excised inside-out patch, STREX:S24E channels expressed in HEK293 cells at +40mV. Panel a shows recordings in normoxic solution, hypoxia and washout. The example time course of the response is illustrated in panel b.

Figure 5.7
Hypoxia does not cause any significant changes in Po of STREX-S24A

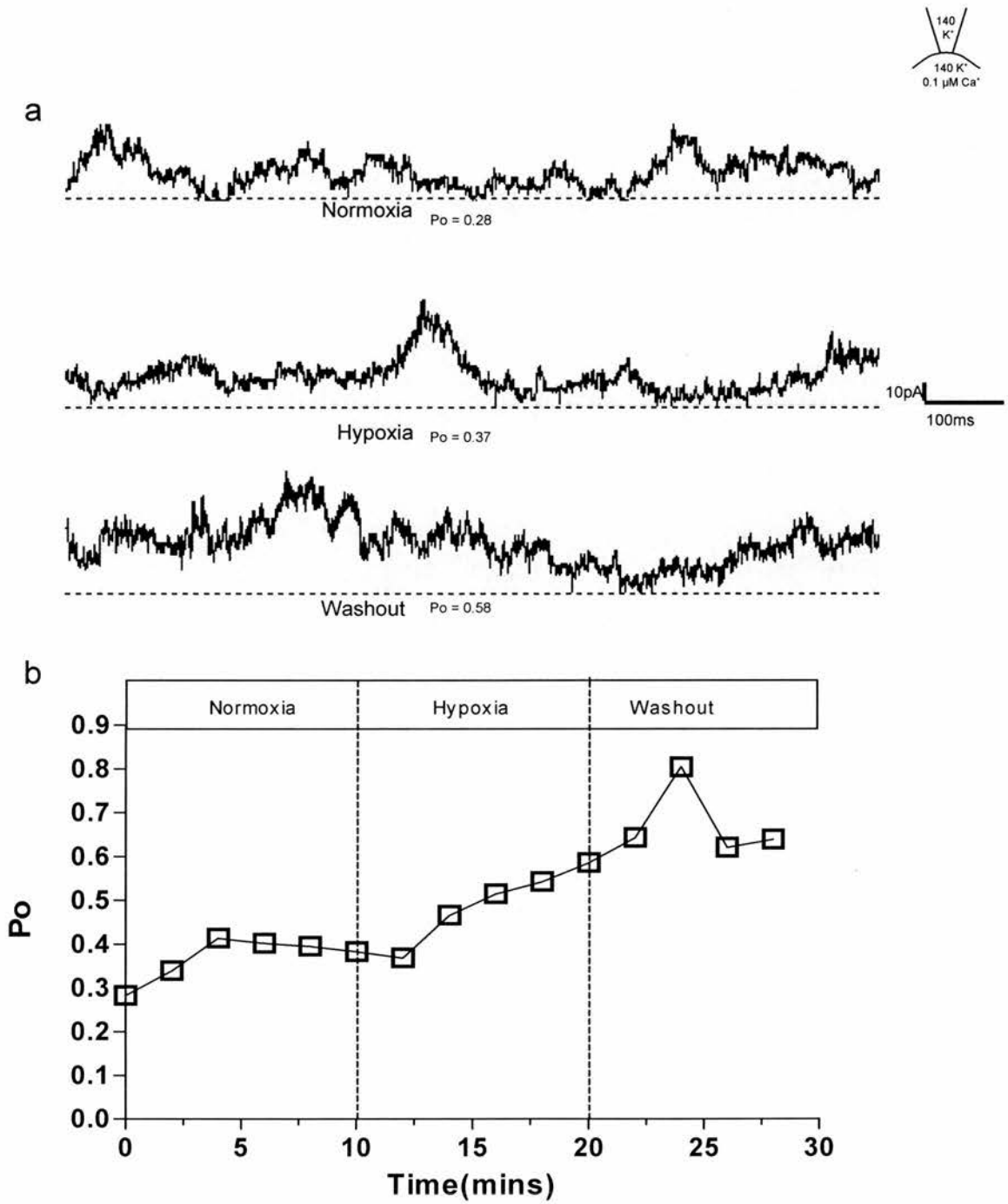


Figure 5.7 Example recordings of single channel activity in an excised inside-out patch, STREX-S24A channels expressed in HEK293 cells at +40mV. Panel a shows recordings in normoxic solution, hypoxia and washout. The example time course of the response is illustrated in panel b.

Figure 5.8
Hypoxia does not cause any significant changes in Po of STREX-R21A

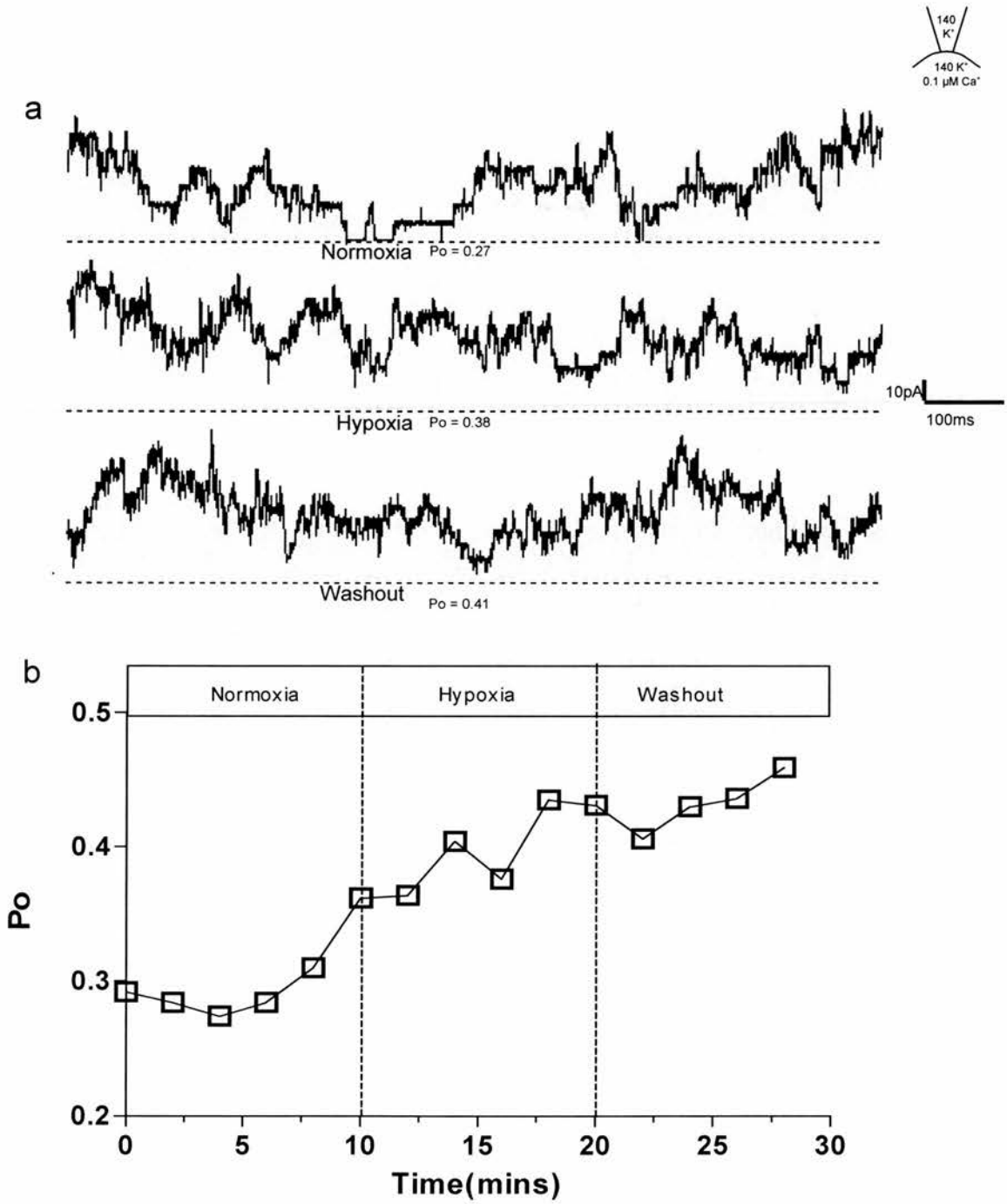
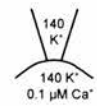


Figure 5.8 Example recordings of single channel activity in an excised inside-out patch, STREX-R21A channels expressed in HEK293 cells at +40mV. Panel a shows recordings in normoxic solution, hypoxia and washout. The example time course of the response is illustrated in panel b.

Figure 5.9
Summary of STREX and mutant channels response to changes in oxygen tension



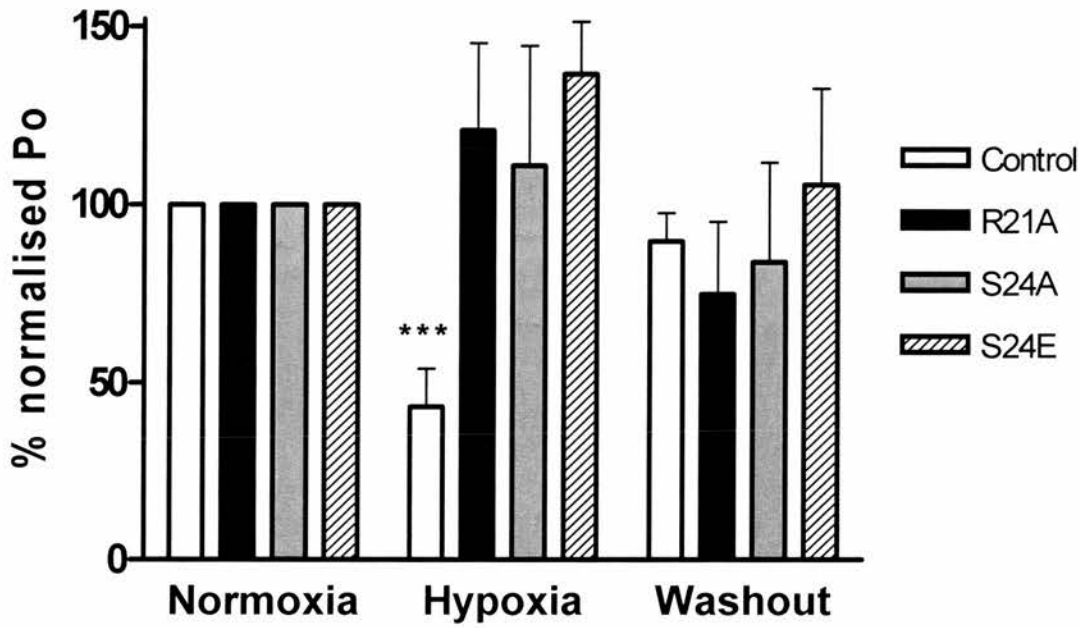


Figure 5.9 Summary graph showing the mean STREX responses to hypoxia compared to those of the STREX mutants, R21A, S24A and S24E. The mutants do not show significant changes in Po that correlate with changes in oxygen tension. Data mean ± sem n = 5, *** = $p < 0.001$ Kruskal-Wallis with Dunns posthoc test compared to normoxic control.

5.2.3 Utilising AMP-PNP, a non-hydrolysable ATP analogue, to investigate the response of STREX channels to hypoxia

The mutations made at the putative phosphorylation site elucidated that the RxCS site was indeed necessary for the response of STREX channels to hypoxia. However, it was inconclusive in proving if direct phosphorylation of the S24 amino acid was necessary. Magnesium is an absolute requirement of phosphorylation so STREX hypoxia experiments were conducted in 1 mM intracellular ATP but in the absence of magnesium to probe if ATP could still modulate the response of STREX channels to hypoxia. The stability of the magnesium free inside-out patches was poor hence it was difficult to make them last the full duration of the experiment. In n = 3 experiments that were successfully completed the hypoxic response of STREX channels was variable, one patch showed an increase in channel activity (80%) upon decrease in oxygen tension, one showed a decrease (75%) whilst another did not respond. The trend was similar to those of the STREX hypoxia experiments conducted in the presence of 1 mM ATP and magnesium, due to the fragility of the patches the data were not viable but suggest that perhaps a phosphorylation-independent method could be the mechanism of STREX hypoxic modulation.

This suggests that ATP may mediate an effect independent of phosphorylation. To further explore this role of ATP in the hypoxia response 5'-adenylylimidodiphosphate (AMP-PNP) tetralithium salt was used. AMP-PNP is a poorly hydrolysable analogue of ATP that can not be used as a phosphate donor by protein kinases to phosphorylate target proteins. Therefore any changes seen in the presence of AMP-PNP could be attributed to phosphorylation-independent modulation of the STREX channel itself.

Patch clamp experiments were carried out as before, with the excised patch immediately exposed to 1 mM AMP-PNP for 10 minutes in normoxia before perfusion with hypoxic solution in the continued presence of AMP-PNP and then washout. Although AMP-PNP did not elicit any significant changes in the absolute Po of STREX, the single channel amplitude of STREX was significantly reduced in the presence of AMP-PNP than in the absence (at +40 mV unitary current amplitude was 9.3 ± 0.1 pA and 10.5 ± 0.1 pA respectively, $p<0.001$ Unpaired t-test), ATP did not mediate any such effect on single channel amplitude. The response of STREX channels to hypoxia in the presence of AMP-PNP was variable (Figure 5.10) out of 3 patches, one showed increased channel activity, one decreased, whilst one did not respond to changes in

channel activity. STREX hypoxia responses conducted in parallel in the absence of AMP-PNP showed a robust reversible inhibition of channel activity during hypoxia (55 ± 8 % mean inhibition of channel activity, $p<0.001$ Kruskal-Wallis with Dunns posthoc test n = 15). AMP-PNP does not consistently affect channel behaviour during low oxygen tension in comparison to STREX control. The potential modulation of the response of STREX channels to hypoxia seen in the presence of AMP-PNP is similar to the ATP experiments (in the presence of magnesium), however now there is an additional unresponsive group as with the magnesium free ATP experiments suggesting that the most probable mode of action for ATP modulation of the response of STREX channels to hypoxia is phosphorylation-independent.

Figure 5.10
Response of STREX channels to hypoxia in the presence of AMP-PNP

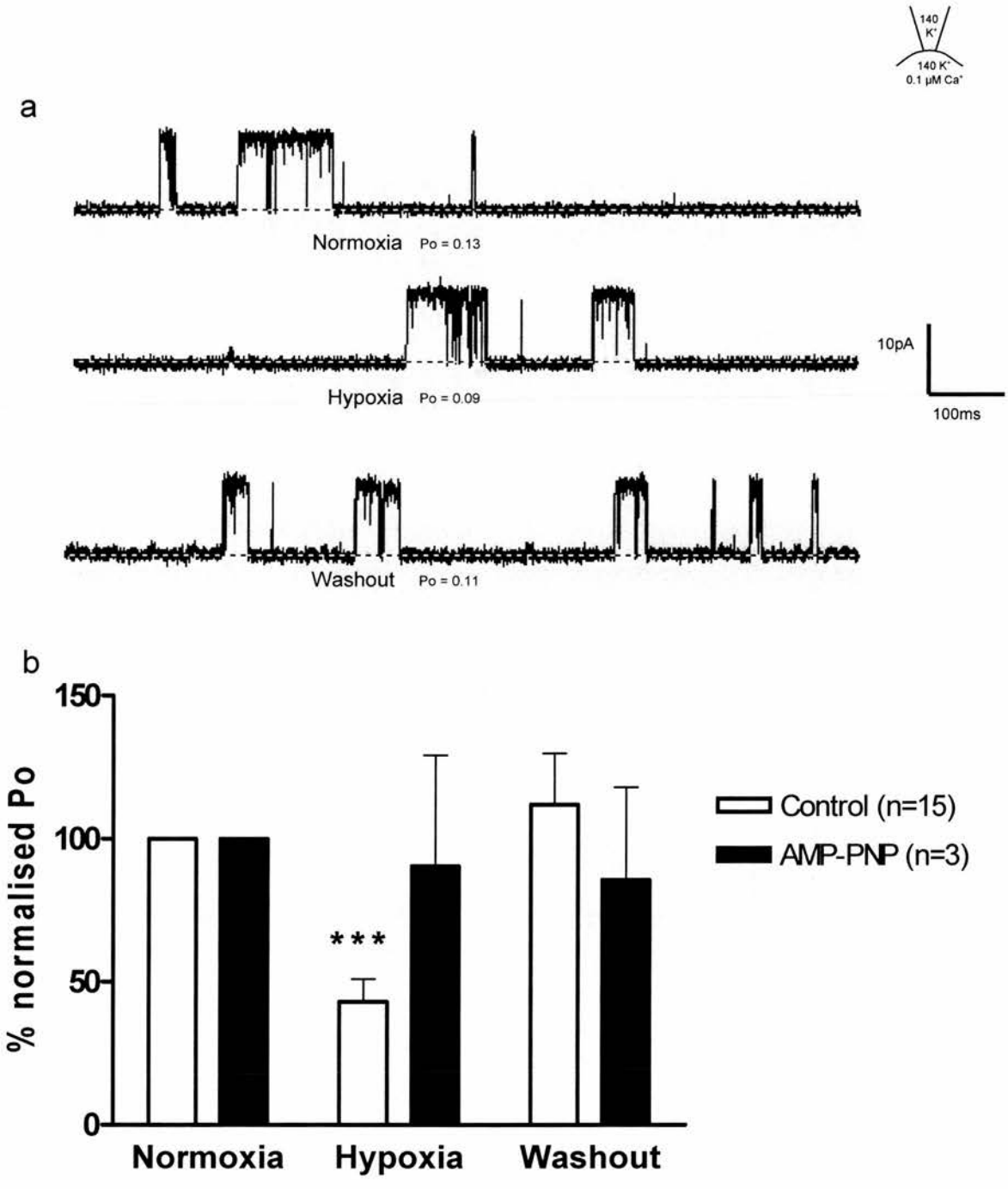


Figure 5.10 Example recordings of single channel activity in an excised inside-out patch in 1mM AMP-PNP at the extracellular face, of STREX channels transiently expressed in HEK293 cells at +40mV. Panel a shows control recordings in normoxic solution, hypoxia and washout phase with normoxic solution, Po values are indicated. The summary bar chart illustrates the variability of the response in the presence of AMP-PNP. Data mean ± sem with n numbers indicated, Kruskal-Wallis test with Dunns posthoc (*** = p0.001).

5.2.4 Lithium blocks the response of STREX channels to hypoxia

The effect of AMP-PNP on STREX channel activity was variable under conditions of low oxygen tension, in addition to the activation and inhibition responses on STREX channel activity induced (as with ATP treatment) an unresponsive group was also revealed. As the AMP-PNP used was a tetra-lithium salt it could be that the lithium salt itself may have induced the non-responsive phenotype, therefore it was necessary to conduct control experiments to eliminate lithium-mediated effects. To address whether the presence of 4 mM lithium at the intracellular face of the patch modified the response of STREX channels to hypoxia, recordings were made under identical conditions as control STREX hypoxia experiments on the same batch of transfected HEK293 cell, except that the intracellular face of the patch was exposed to 4 mM lithium immediately upon patch excision and maintained throughout the experiment. Surprisingly the presence of lithium at the intracellular face of the patch prevented the typical hypoxia induced decrease in STREX channel activity (Figure 5.11 & 5.14). The changes in Po were quite variable (Figure 5.14) but there is a significant increase in channel activity, upon perfusion with hypoxic solution in the presence of lithium (60 ± 35 % mean

activation of channel activity, $p<0.001$ 2-way ANOVA with Bonferroni posthoc test). Normoxia control experiments conducted in the presence of 4 mM intracellular lithium, parallel to the hypoxia experiments, did not show any significant changes in channel activity (Figure 5.14).

The absence of inhibition of STREX channels activity in response to hypoxia could be attributed to the monovalent cation effect of lithium; hence STREX hypoxia experiments were conducted in parallel under identical conditions in the presence of 4 mM additional sodium at the intracellular face. Upon perfusion of hypoxic solution a decrease in channel activity is observed (Figure 5.12 & 5.14) that is not significantly different to the control STREX hypoxia response in the absence of additional cations (60 ± 8% mean inhibition of channel activity, $p<0.001$ 2-way ANOVA with Bonferri posthoc test). Although the channel inhibition in response to a decrease in oxygen tension is not reversible in the presence of additional sodium which implies that there is an additional effect that sodium is mediating, perhaps inducing a structural change on the channel protein itself thereby preventing it to return back to its normal conformation? There were no significant changes in channel activity under control normoxic conditions in the presence of sodium (Figure 5.14).

Figure 5.11
Lithium blocks the response of STREX channels to hypoxia

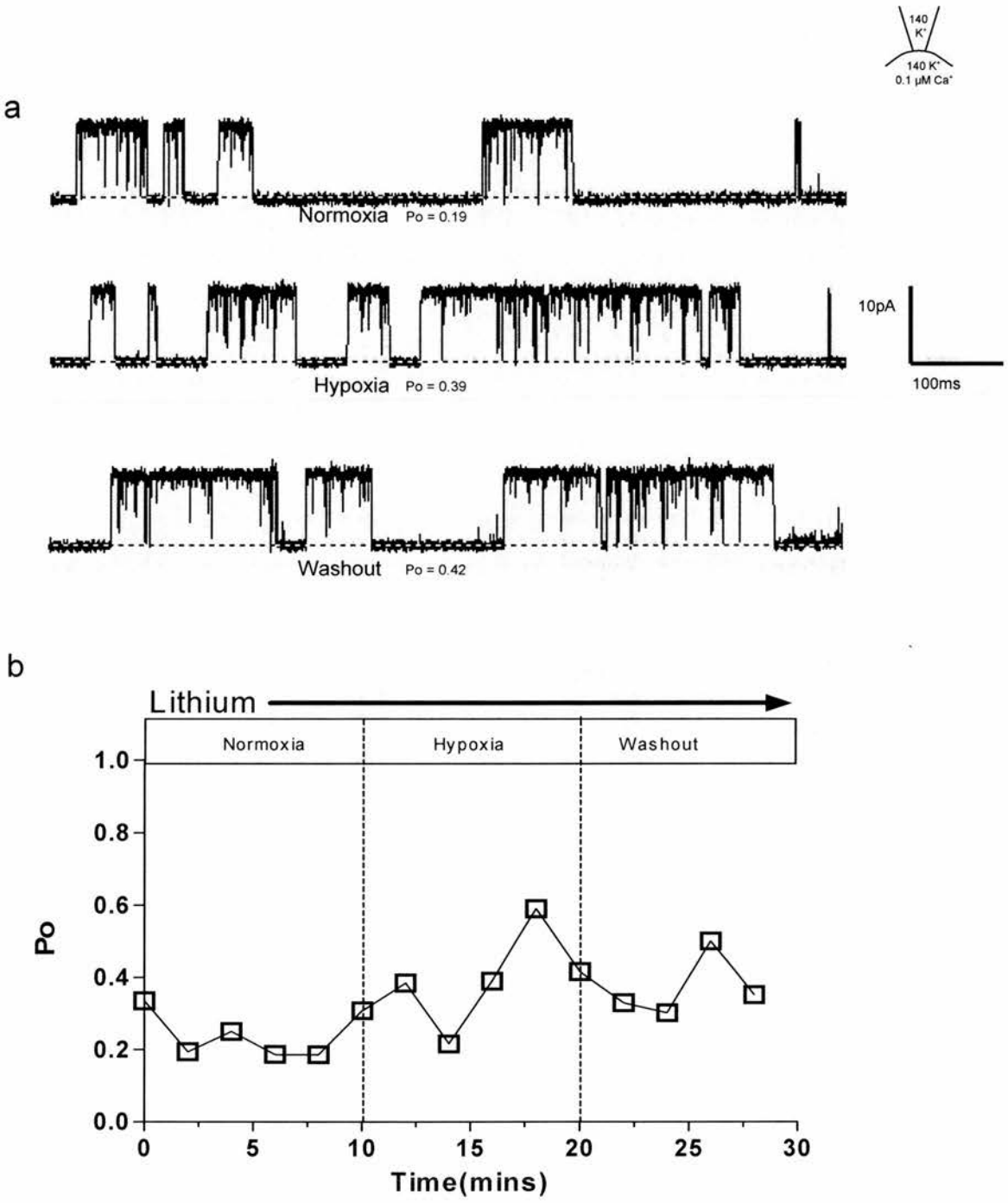


Figure 5.11 Example recordings of single channel activity in an excised inside-out patch in 4mM lithium at the extracellular face, of STREX channels transiently expressed in HEK293 cells at 40mV. Panel a shows control recordings in normoxic solution, hypoxia and the washout phase with normoxia solution, Po values are indicated. The example time course of the response is illustrated in panel b.

Figure 5.12
Additional sodium does not affect the response of STREX channels to hypoxia

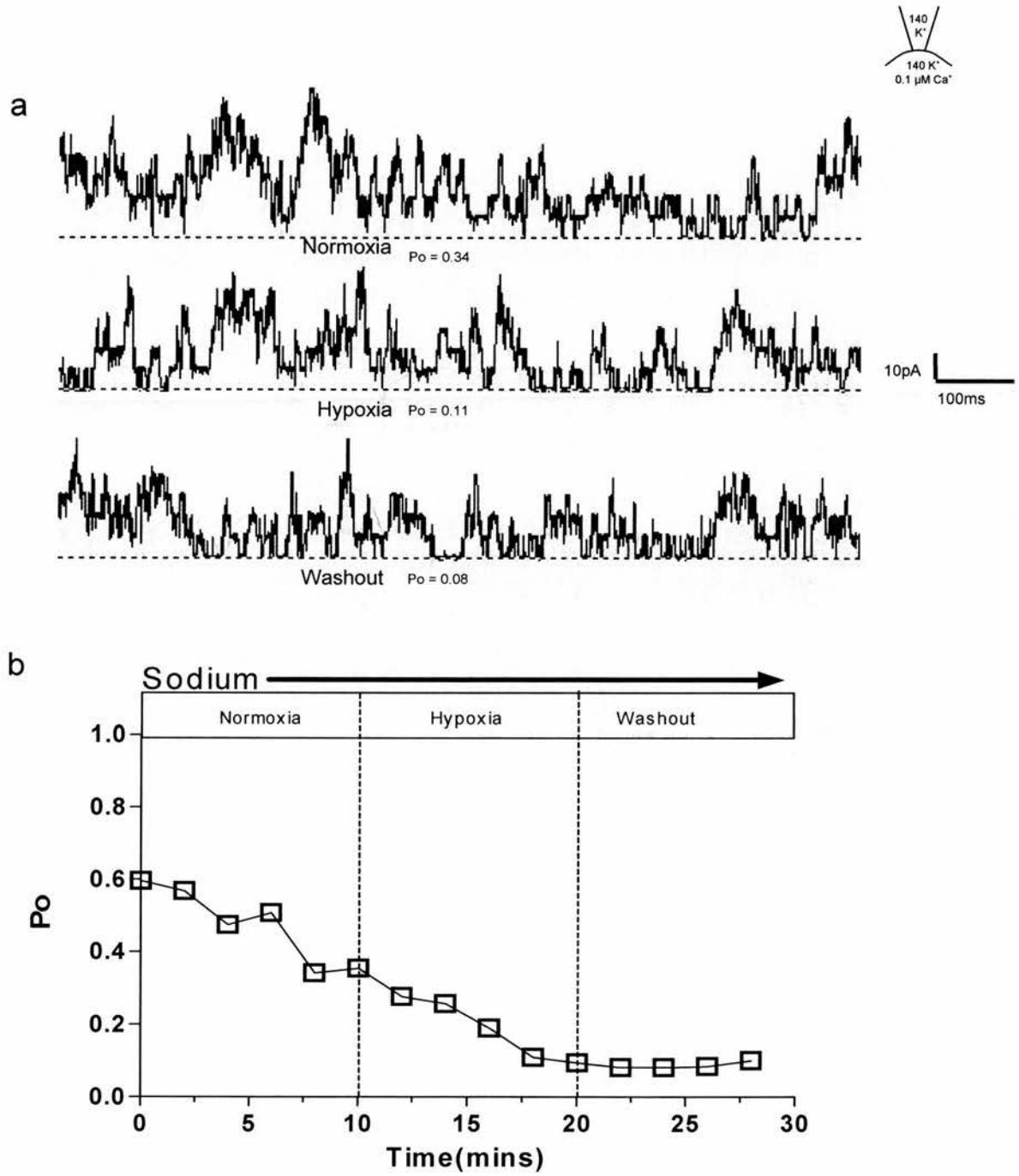


Figure 5.12 Example recordings of single channel activity in an excised inside-out patch with 4mM additional sodium at the intracellular face, of STREX channels transiently expressed in HEK293 cells at 40mV. Panel a shows control recordings in normoxic solution, hypoxia and the washout phase with normoxia solution, Po values are indicated. The example time course of the response is illustrated in panel b.

5.2.5 Magnesium modulates the response of STREX channels to hypoxia

Lithium competes for magnesium binding sites (Mota de Freitas et al., 2006), in that it mimics the behaviour of magnesium as they share physicochemical properties. An interactive mechanism between lithium and magnesium for magnesium-binding sites could be the underlying mode of action for the lithium block of the inhibition of STREX channels to hypoxia. It would therefore be rational to assume that if additional magnesium was administered in place of lithium at the intracellular face of the patch then the effects on channel activity under conditions of low oxygen tension would be similar.

To address the hypothesis of whether the presence of magnesium at the intracellular face of the patch, modified the response of STREX channels to hypoxia, channels were recorded under identical conditions, except that the intracellular face of the patch was exposed to 4 mM additional magnesium immediately upon patch excision and maintained throughout the experiment. The presence of additional magnesium caused an oscillatory effect on STREX channel activity throughout the timecourse of the experiments (Figure 5.13), the oscillation seen in all patches did not correlate with changes in oxygen tension.

Magnesium appeared to modify channel behaviour but not in a way that was consistent or sustained, an effect which was independent of changes in oxygen tension. So, unlike the effect in the presence of the magnesium-mimetic, lithium, the affect of hypoxia on STREX channels in the presence of magnesium itself is variable. There is an absence of the inhibition of STREX channels to hypoxia in the presence of magnesium (Figure 5.14a), channel activity significantly increases under conditions of low oxygen tension (18 ± 8 % mean activation of channel activity $p<0.05$ 2-way ANOVA with Bonferri posthoc test). However this increase in channel activity is also observed under normoxic conditions (30 ± 18 % mean activation of channel activity, Figure 5.14b) implying that the magnesium effect on channel Po is independent of changes in oxygen tension and most probably due to a structural change induced by additional magnesium in the intracellular face of the patch.

Figure 5.13
Magnesium does not cause a decrease in STREX channel activity during hypoxia

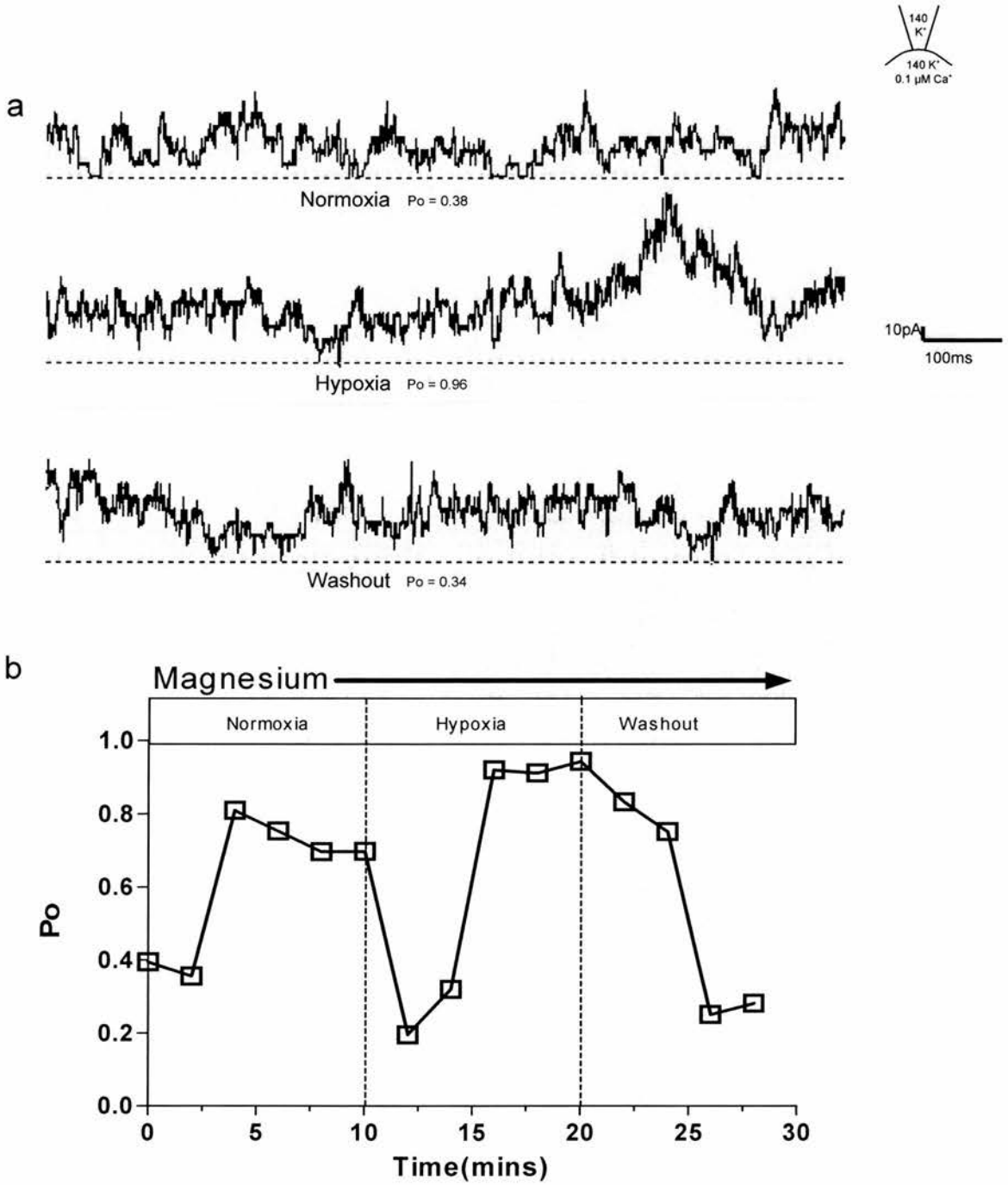


Figure 5.13 Example recordings of single channel activity in an excised inside-out patch with 4mM additional magnesium at the intracellular face, of STREX channels transiently expressed in HEK293 cells at 40mV. Panel a shows control recordings in normoxic solution, hypoxia and the washout phase with normoxia solution, Po values are indicated. The example time course of the response is illustrated in panel b.

Figure 5.14
Effect of cations on the response of STREX channels to hypoxia

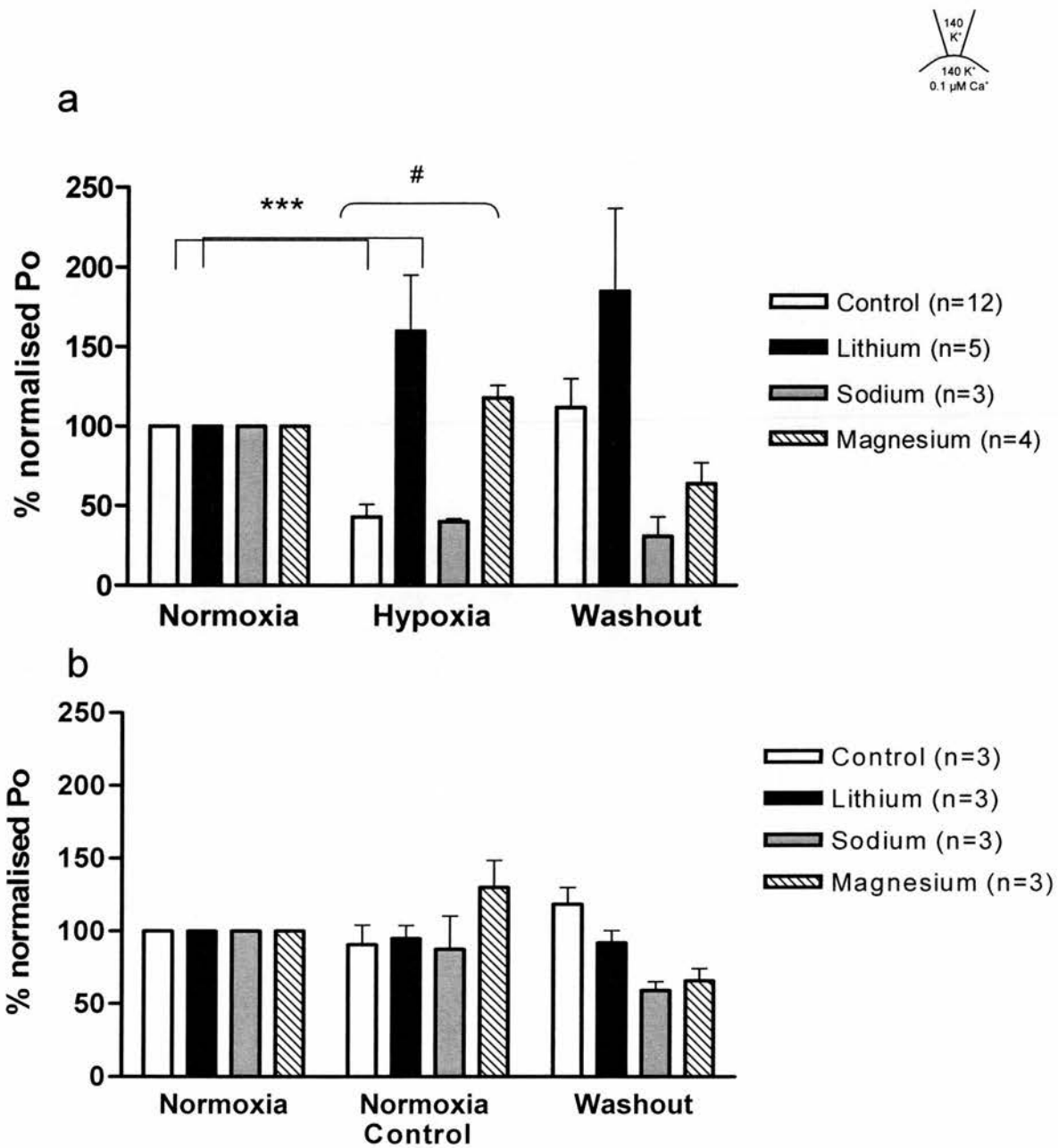


Figure 5.14 Panel a is a summary graph of the mean STREX change in Po to hypoxia with different cations exposed to the intracellular face of the patch. The lithium and sodium responses were statistically significantly different in comparison to the control hypoxia response. Panel b is the summary graph of the control normoxia experiments done in the presence of the different cations to investigate their effect on STREX alone. Data mean ± sem with n numbers indicated, 2way ANOVA with Bonferroni posthoc test (*** ≤ p0.001, # < p 0.05 all significantly different).

5.2.6 Lithium and magnesium reduce single channel amplitude of STREX

In the AMP-PNP STREX hypoxia experiments the single channel amplitude was significantly decreased. Thus it was important to investigate the single channel amplitude of STREX channels in presence of lithium and magnesium at the intracellular surface of the patch. Single channel recordings were made of STREX channels at a range of potentials (-80 mV to +80 mV) on the same batch of transfected HEK293 cells, in the presence of lithium and additional magnesium in excised inside-out patches with 0.33 μM free calcium to investigate any possible effects on single channel amplitude.

Addition of lithium at the intracellular face of an excised patch significantly decreased STREX single channel amplitude with no significant changes to channel Po (Figure 5.15). This decrease in single channel amplitude is only seen at positive potentials (Figure 5.16) and was reversible upon washout (from 9.8 ± 0.3 pA to 8.0 ± 0.2 pA and 9.3 ± 0.2 pA at +40 mV in control 2 mM magnesium, additional 4 mM lithium and washout with 2 mM magnesium respectively, $p<0.001$ ANOVA plus Tukey posthoc test).

Figure 5.15
Lithium decreases single channel amplitude of STREX channels

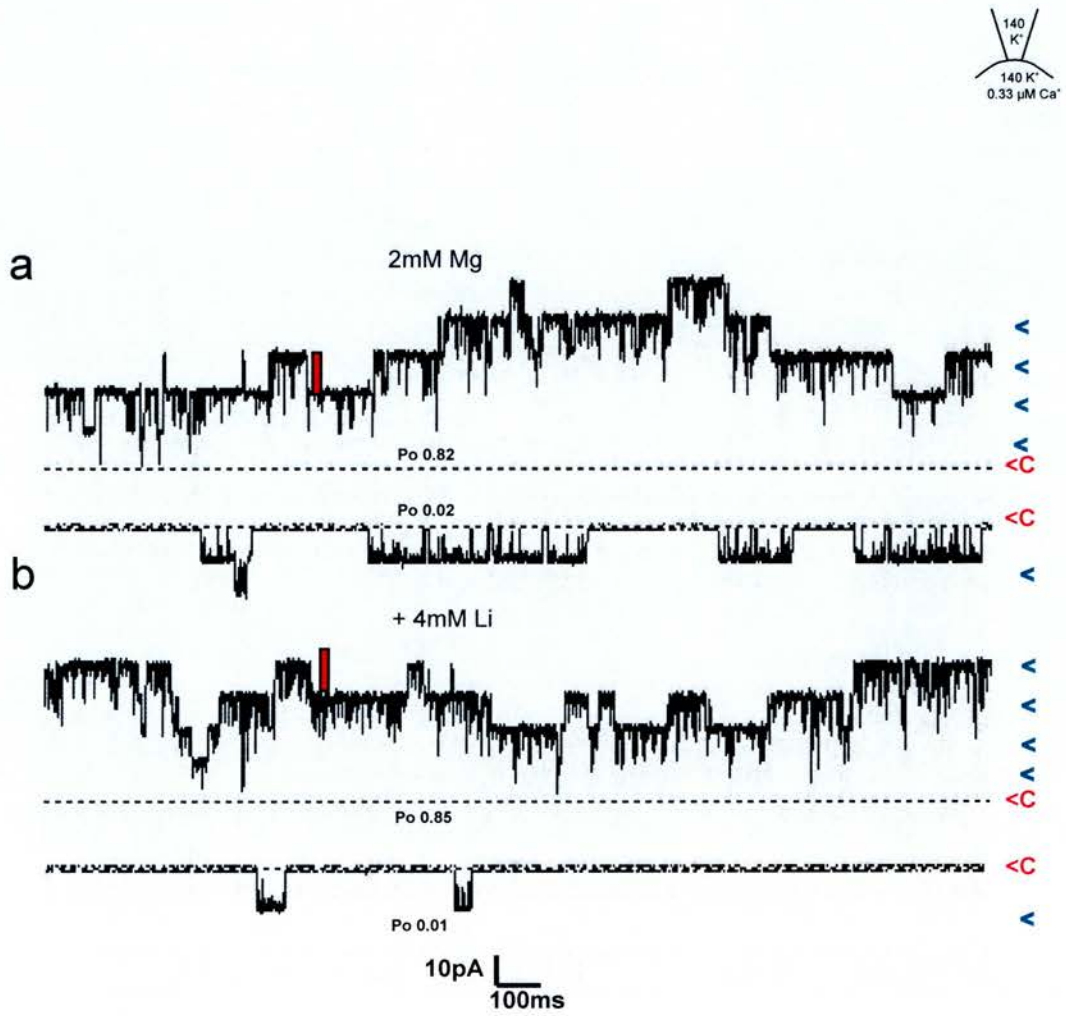
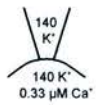


Figure 5.15 In excised inside out patches, addition of 4mM lithium to the control 2mM magnesium solution at the intracellular surface reduced single channel amplitude. Representative single channel traces at +/- 40mV in 0.33µM [Ca2+] are shown (panel a is 2mm magnesium and b with the addition of lithium, C indicates closed state and > showing open states). ▌ Symbol indicates 10pA on the traces, to compare the change in the single channel amplitude upon addition of lithium. The Po values are shown on the traces and display no significant change upon addition of lithium.

Figure 5.16
Lithium reduces single channel amplitude of STREX channels



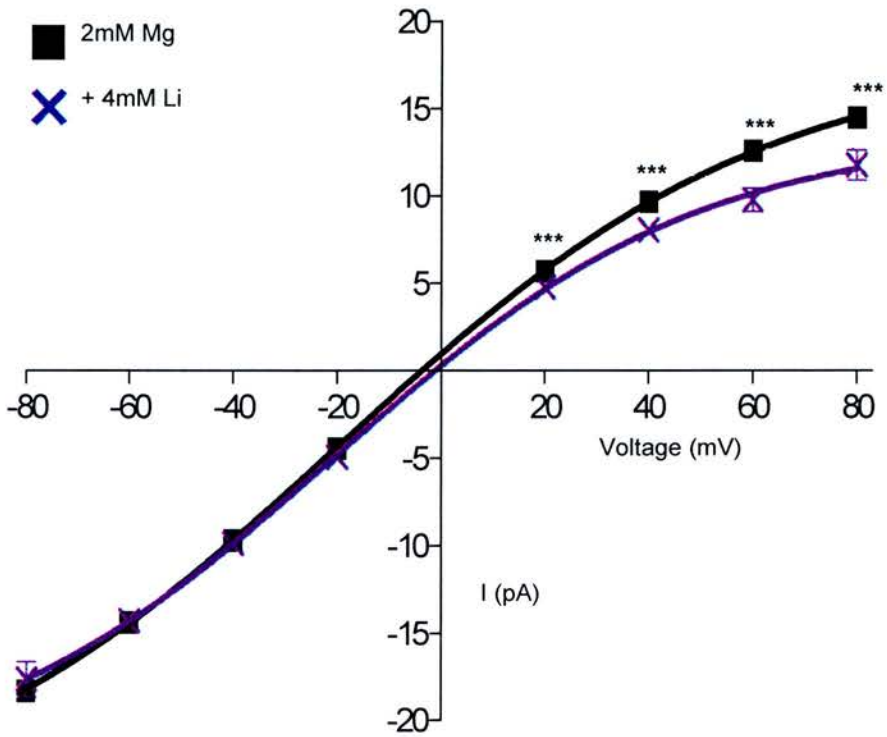


Figure 5.16 In excised inside out patches, addition of 4mM lithium to the control 2mM magnesium solution at the intracellular surface signifiacntly reduced single channel amplitude. The effect was voltage dependent as the decrease in channel amplitude was only observed at depolarized potentials (+20mV to +80mV), and was reversible upon washout. Data are mean ± s.e.m, n>3 ***p≤0.001 compared to other treatment group, ANOVA plus Tukey posthoc.

To further investigate the effect of magnesium on STREX single channel activity, excised inside-out patches of STREX channels were first exposed to 0 mM magnesium, then 2 mM magnesium (normal control bath solution) and then 6 mM (Figure 5.17). Increases in magnesium concentration caused a significant decrease in single channel amplitude. In the absence of intracellular magnesium the single unitary conductance at 40 mV was 10.0 ± 0.3 pA, decreasing to 8.0 ± 0.5 pA and 7.0 ± 0.4 pA at 2 and 6 mM magnesium respectively ($n = 3$, $p<0.001$ ANOVA plus Tukey posthoc test, Figure 5.18). Furthermore, increasing magnesium concentration at the intracellular face of the patch actually caused a significant increase in STREX channel activity (Figure 5.17 & 5.28, 30 ±12 mV increase in $V_{1/2}$ max $p<0.01$ Students Paired t-test).

Magnesium has previously been shown to increase BK channel activity in retinal glial cells (Bringmann *et al.*, 1997) and to enhance apparent calcium sensitivity of BK channels in mammalian skeletal muscle (Golowasch *et al.*, 1986) but this is the first instance that magnesium and lithium modulation has been shown on the STREX splice variant of the BK channel. STREX channels are the more calcium sensitive variant of BK channels which could be why it possesses this enhanced sensitivity to lithium and magnesium. The less calcium sensitive BK

ZERO variant was thus used to investigate if the changes in single channel amplitude seen with magnesium and lithium were due to the presence of the STREX 59 amino acid insert. ZERO channels also showed a significant decrease in single channel amplitude in the presence of lithium and increasing concentrations of magnesium (Figure 5.19 & 5.20) again only at positive potentials. This implies that the changes in conductance observed are independent of the STREX insert and potentially due to structural changes induced by these cations elsewhere in the channel. Nonetheless the lack of inhibition of STREX channels by hypoxia in the presence of these cations could still be attributable to the changes in channel behaviour elicited in the presence of magnesium and lithium.

Figure 5.17
Magnesium decreases single channel amplitude of STREX channels

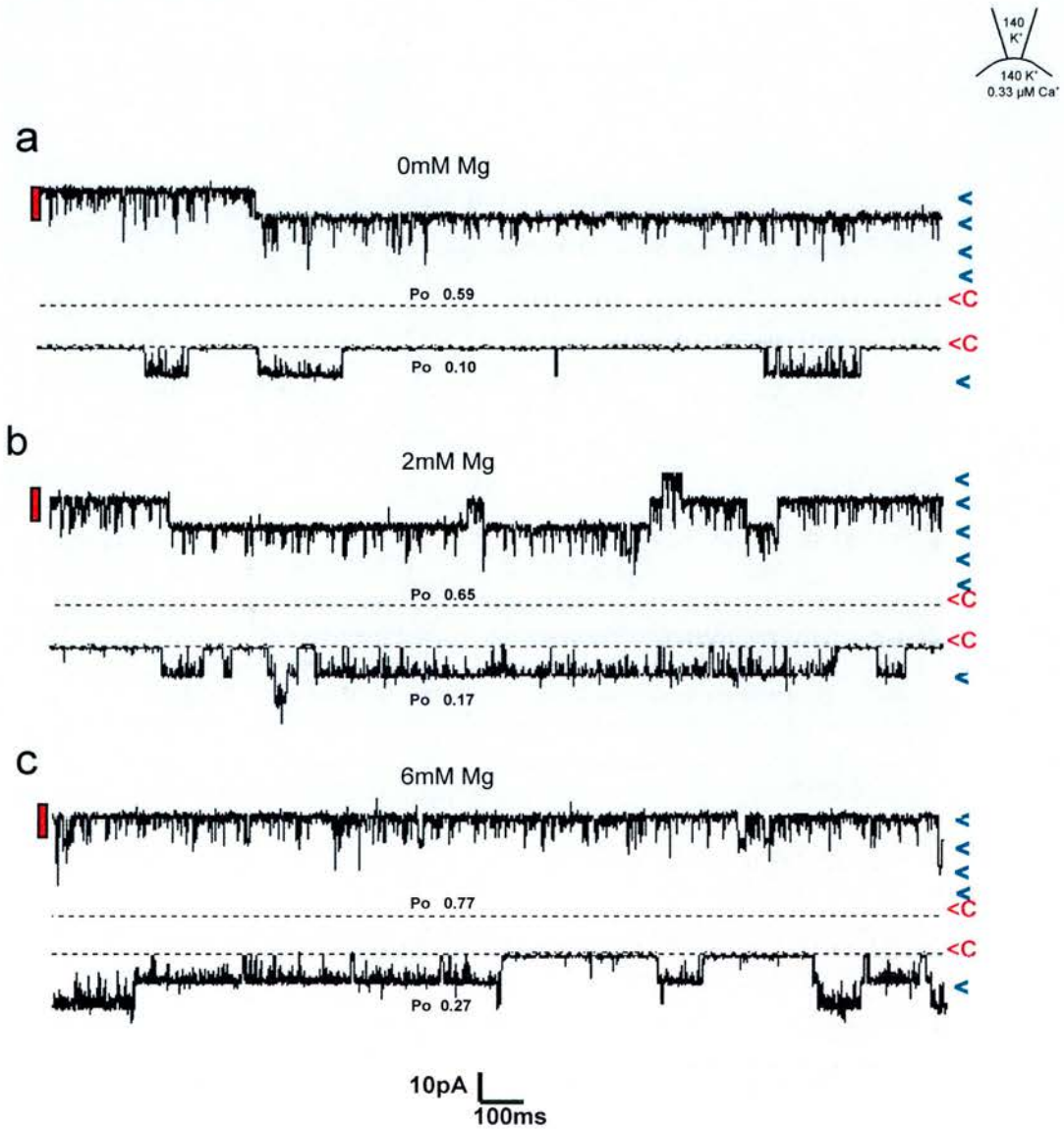
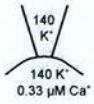


Figure 5.17 In excised inside out patches of HEK-293 cells transiently expressing STREX, increasing the concentration of magnesium at the intracellular face reduced the single channel amplitude Representative single channel traces at +/- 40mV in 0.33µM [Ca2+] are shown (panel a is 0mM magnesium, b is 2mM magnesium and c is illustrating the response in 6mM magnesium and b with the addition of lithium, C indicates closed state and > showing open states). Symbol ▌ indicates 10pA on the traces, to compare the change in the single channel amplitude upon addition of increasing magnesium. The Po values are shown on the traces and display a significant increase in channel activity as the concentration of magnesium is increased.

Figure 5.18
Magnesium reduces single channel amplitude of STREX channels



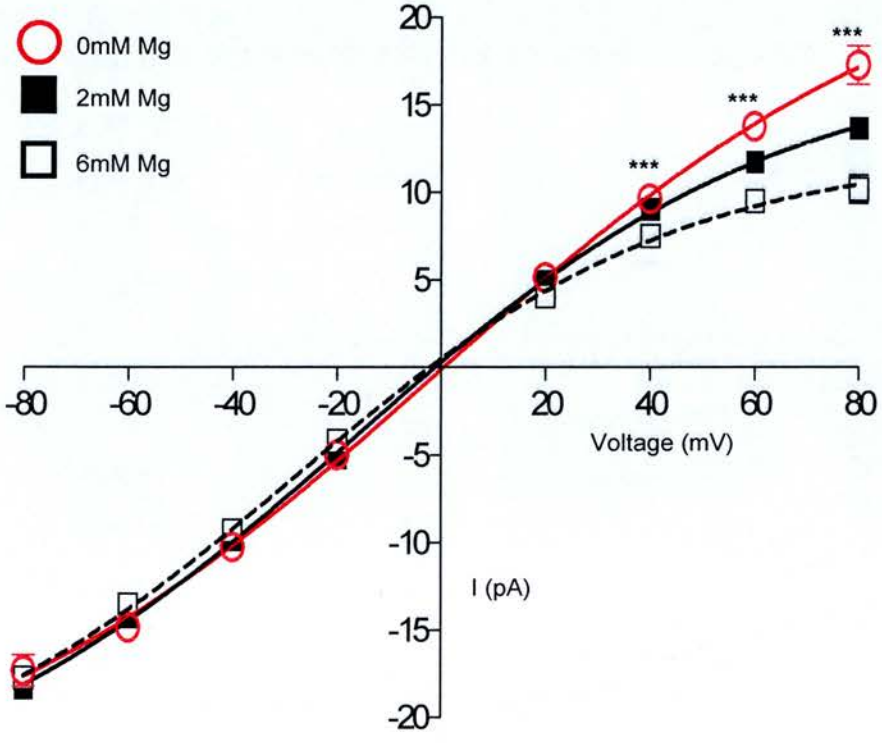


Figure 5.18 HEK-293 cells were transiently transfected with STREX. In excised inside out patches an increase in the concentration of magnesium at the intracellular surface significantly reduced single channel amplitude. The effect was voltage dependent as the decrease in channel amplitude was only observed at depolarized potentials (+20mV to +80mV). Data are mean ± s.e.m, n=3 *** $p<0.001$ compared to other treatment groups, ANOVA plus Tukey post-hoc.

Figure 5.19
Lithium reduces single channel amplitude of ZERO channels

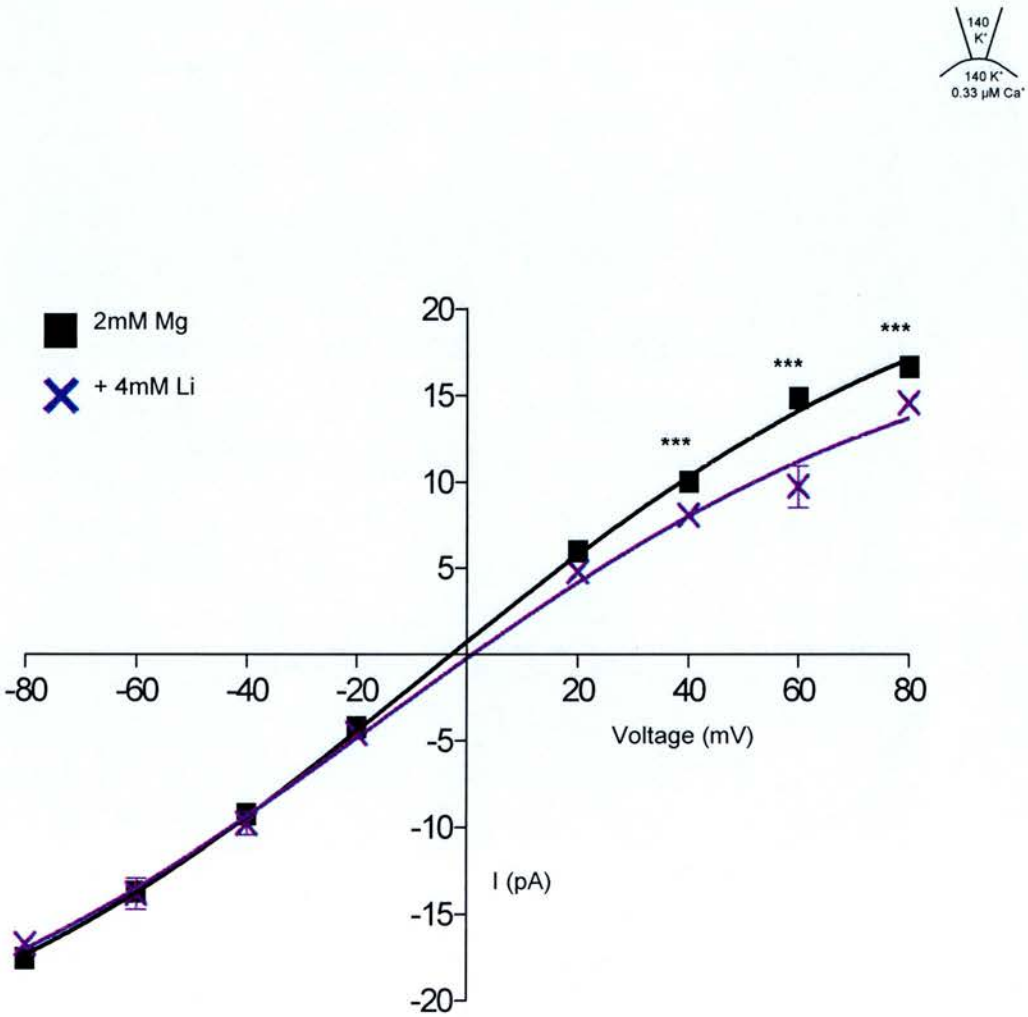
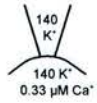


Figure 5.19 HEK-293 cells were transiently transfected with the ZERO BK channel variant. In excised inside out patches, addition of 4mM lithium to the control 2mM magnesium solution at the intracellular surface significantly reduced single channel amplitude. The effect was voltage dependent as the decrease in channel amplitude was only observed at depolarized potentials (+20mV to +80mV), and was reversible upon washout (data not shown). Data are mean ± s.e.m, n=4 ***p≤0.001 compared to other treatment group, ANOVA plus Tukey posthoc.

Figure 5.20
Magnesium reduces single channel amplitude of ZERO channels



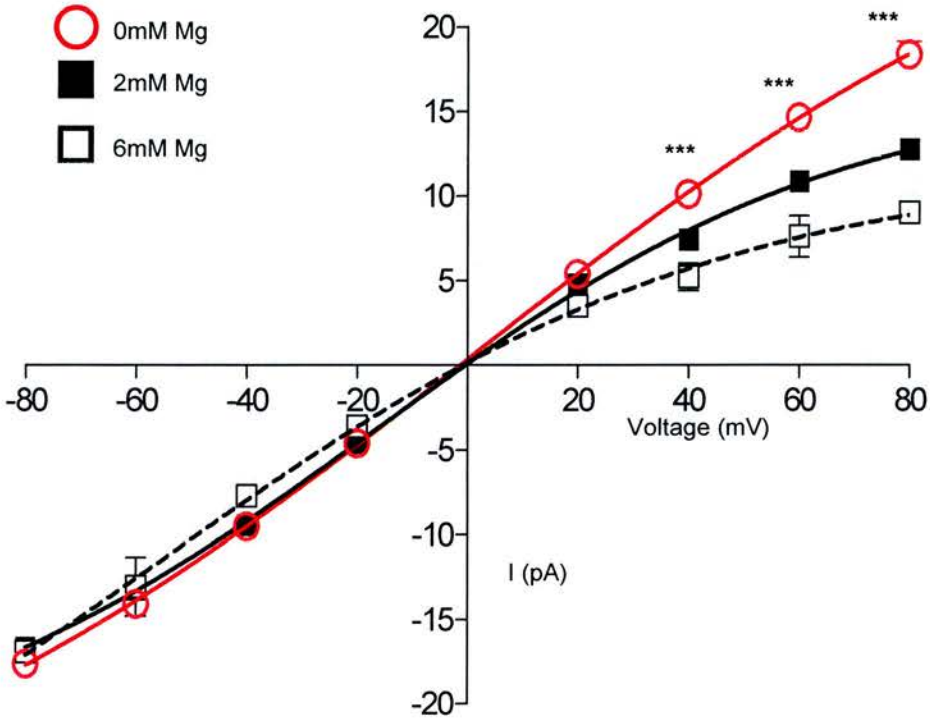


Figure 5.20 HEK-293 cells were transiently transfected with the ZERO BK channel variant. In excised inside out patches an increase in the concentration of magnesium at the intracellular surface significantly reduced single channel amplitude. The effect was voltage dependent as the decrease in channel amplitude was only observed at depolarized potentials (+20mV to +80mV). Data are mean ± s.e.m, n=3 *** $p<0.001$ compared to other treatment groups, ANOVA plus Tukey posthoc.

Figure 5.21
High affinity magnesium binding site E374 in STREX channels

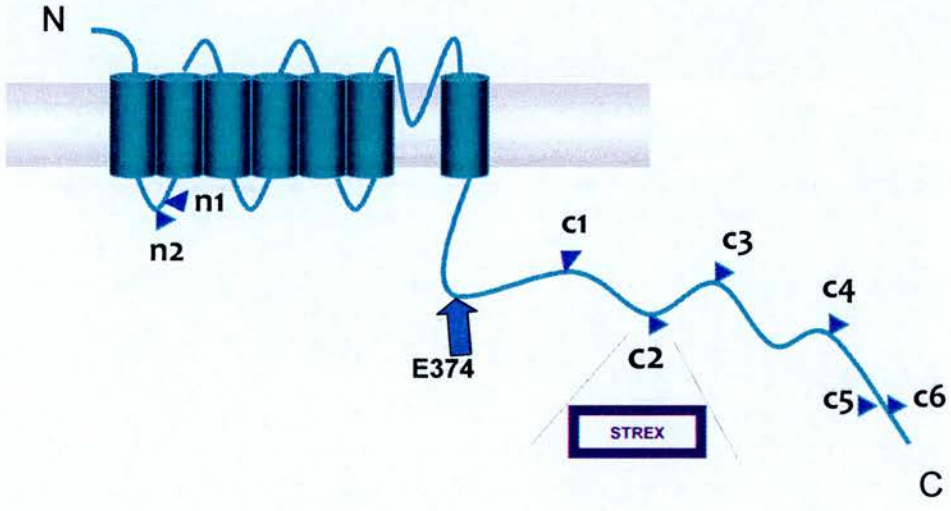


Figure 5.21 The putative high affinity magnesium binding site is indicated in the diagram above. Mutation of this site from a glutamine to an alanine was used to investigate the role of this site in the reduction of the single channel conductance and the change in Po.

5.2.7 The high-affinity magnesium binding site E374, does not contribute to the regulation of BK single channel activity by lithium and magnesium

Magnesium has previously been shown to activate BK channels (Golowasch *et al.*, 1986; Bringmann *et al.*, 1997) and activation can be seen in the presence and absence of calcium (Shi & Cui, 2001; Zhang *et al.*, 2001). This implies that magnesium activation is independent of any calcium binding to the channel. It was found that mutation of a high-affinity magnesium binding site (E374) completely abolished the magnesium-dependent increase in channel activity (Yang *et al.*, 2006). Therefore it is possible that not only the changes in Po induced by magnesium, but indeed the changes in single channel amplitude caused by magnesium and lithium, shown here for the first time, might be attributed to this high-affinity magnesium binding site E374 (Figure 5.21).

Studies to elucidate the magnesium binding site used chimeric channels (Shi *et al.*, 2002), expressing a Slo1 core and Slo3 tail. The chimeric channels did not contain the calcium bowl but were still sensitive to magnesium activation indicating that the site may be located in the RCK domain (regulator of

conductance of K^+). Sequence alignment of the RCK domain in Slo1 and Slo3 revealed several acidic amino acids present in Slo1 and not Slo3. The residues conserved in Slo1 (mSlo1 and dSlo1) but not in Slo3 were mutated to corresponding amino acids present in Slo3, revealing two hot spots; E374 and E399.

Based on the RCK structure previously proposed (Jiang *et al.*, 2001), it was concluded that magnesium ion binding was co-ordinated by E374, E399 and Q397 (Shi *et al.*, 2002; Xia *et al.*, 2002). Further studies into how magnesium interacted with these sites elucidated that mutation of the E374 and E399 to any amino acid except aspartic acid greatly reduced magnesium sensitivity (Hu *et al.*, 2006b; Yang *et al.*, 2006). However mutation of the Q397 site did not abolish, but modulated the magnesium sensitivity by affecting the electrostatic interaction and conformation of the magnesium binding sites (E374 and E399).

Site-directed mutagenesis and cloning were utilised to make STREX-E374A constructs to investigate the hypothesis that this site could play a role in the magnesium and lithium induced change in STREX channel behaviour. It was

observed that mutation at the E374 site did not affect the reduction in single channel amplitude seen upon addition of 4 mM lithium, however it significantly increased the Po of the channel (Figure 5.22, 5.23 & 5.26 $p<0.01$ Students paired t-test). The reduction in single channel conductance remained the same as the STREX wild type in that it was only seen in positive potentials (Figure 5.23). The STREX-E374A mutant caused a significant decrease in channel amplitude with increasing magnesium concentration as seen before at positive potential, however interestingly a significant decrease in channel activity was now observed at negative potentials (Figure 5.24 & 5.25 $p<0.001$ ANOVA plus Tukey posthoc).

As there is no difference in the single channel responses between STREX wildtype and the E374A mutant channels at positive potentials, the high-affinity magnesium binding site E374 can not be responsible for the changes in the channel amplitude observed in the presence of lithium and magnesium. However it does play a vital role in modulating the channel activity, as the increase in Po observed in wild type STREX with increasing magnesium was abolished with the mutant (Figure 5.24 & 5.26). In STREX wild type, magnesium significantly increased the Po of the channel, as observed in the

leftward shift of $V_{1/2}$ (30 ± 12 mV increase in $V_{1/2}$ max $p<0.01$ Students Paired t-test) in voltage-dependent activation of BK. This was calculated using the Boltzmann equation to fit the activation curves, whereas lithium had no significant effect on channel activity on STREX wildtype channels (Figure 5.26). In contrast, magnesium decreased activity in STREX-E374A producing a rightward shift in $V_{1/2}$ (16 ± 9 mV decrease in $V_{1/2}$ $p<0.01$ Students Paired t-test). Additionally, lithium now produced a significant increase in the channel activity of the mutant (34 ± 18 mV increase in $V_{1/2}$ $p<0.01$ Students Paired t-test). It is therefore possible that the lack of the response of STREX channels to hypoxia in the presence of these cations could be due to the decrease in channel amplitude they induce, which may in turn be responsible for the increase in channel activity seen in their presence.

Figure 5.22
Lithium reduces single channel amplitude in STREX-E374A channel

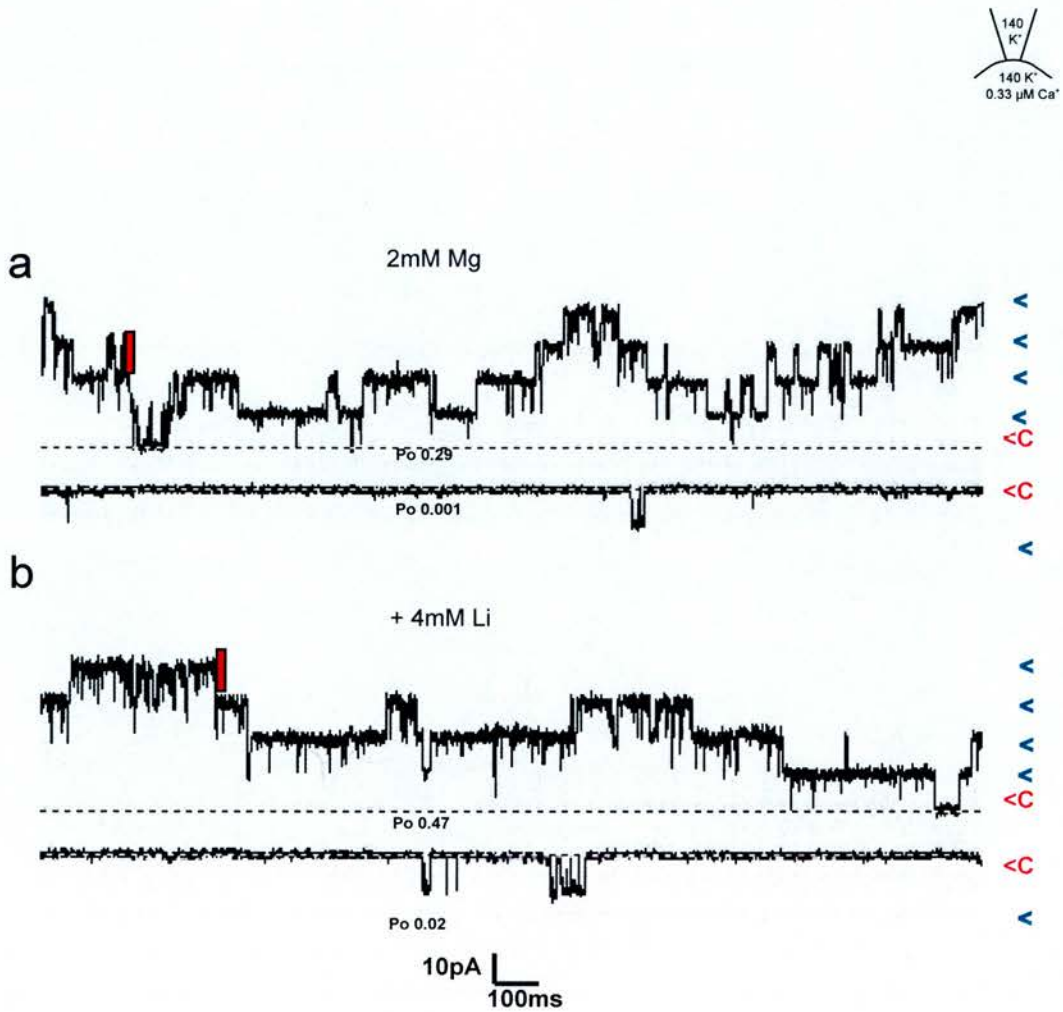
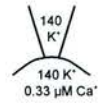


Figure 5.22 In excised inside out patches, addition of 4mM lithium to the control 2mM magnesium solution at the intracellular surface reduced single channel amplitude in STREX-E374A. Representative single channel traces at +/- 40mV in 0.33µM [Ca2+] are shown (panel a is 2mm magnesium and b with the addition of lithium, C indicates closed state and > showing open states). ▮ Symbol indicates 10pA on the traces, to compare the change in the single channel amplitude upon addition of lithium. The Po values are shown on the traces and display no significant change upon addition of lithium.

Figure 5.23
Lithium decreases single channel amplitude of STREX-E374A channel



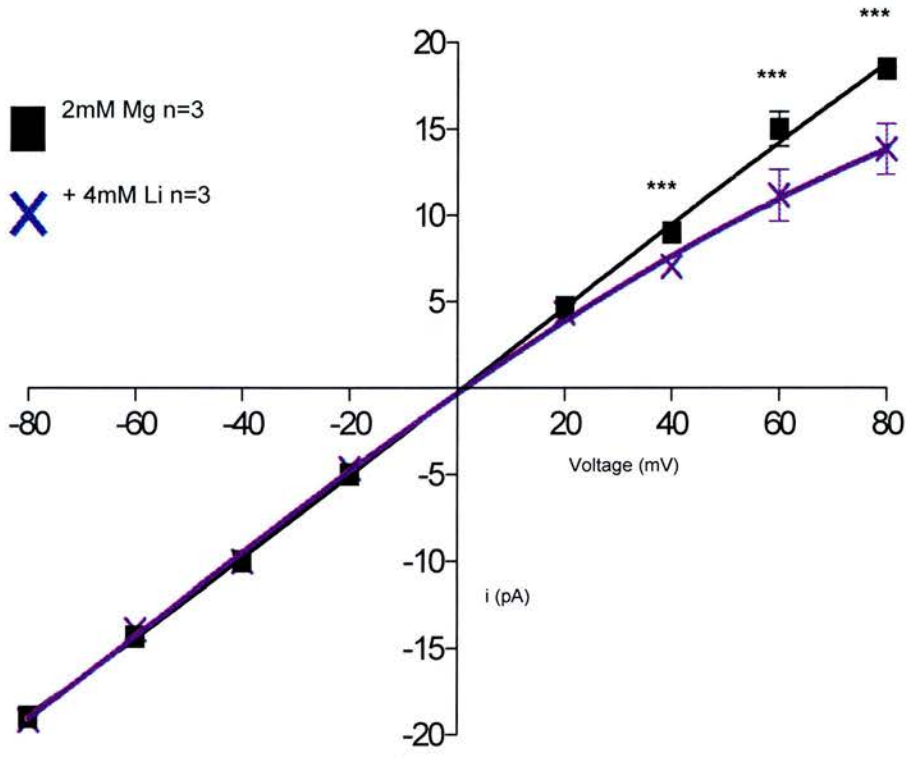


Figure 5.23 Site-directed mutagenesis at the E374 site did not alter the decrease in single channel amplitude caused by lithium at positive potentials in the STREX wildtype. (Data are mean ± s.e.m, *** $p \leq 0.001$ compared to other groups, ANOVA plus Tukey posthoc).

Figure 5.24
Magnesium decreases single channel amplitude in STREX-E374A channel

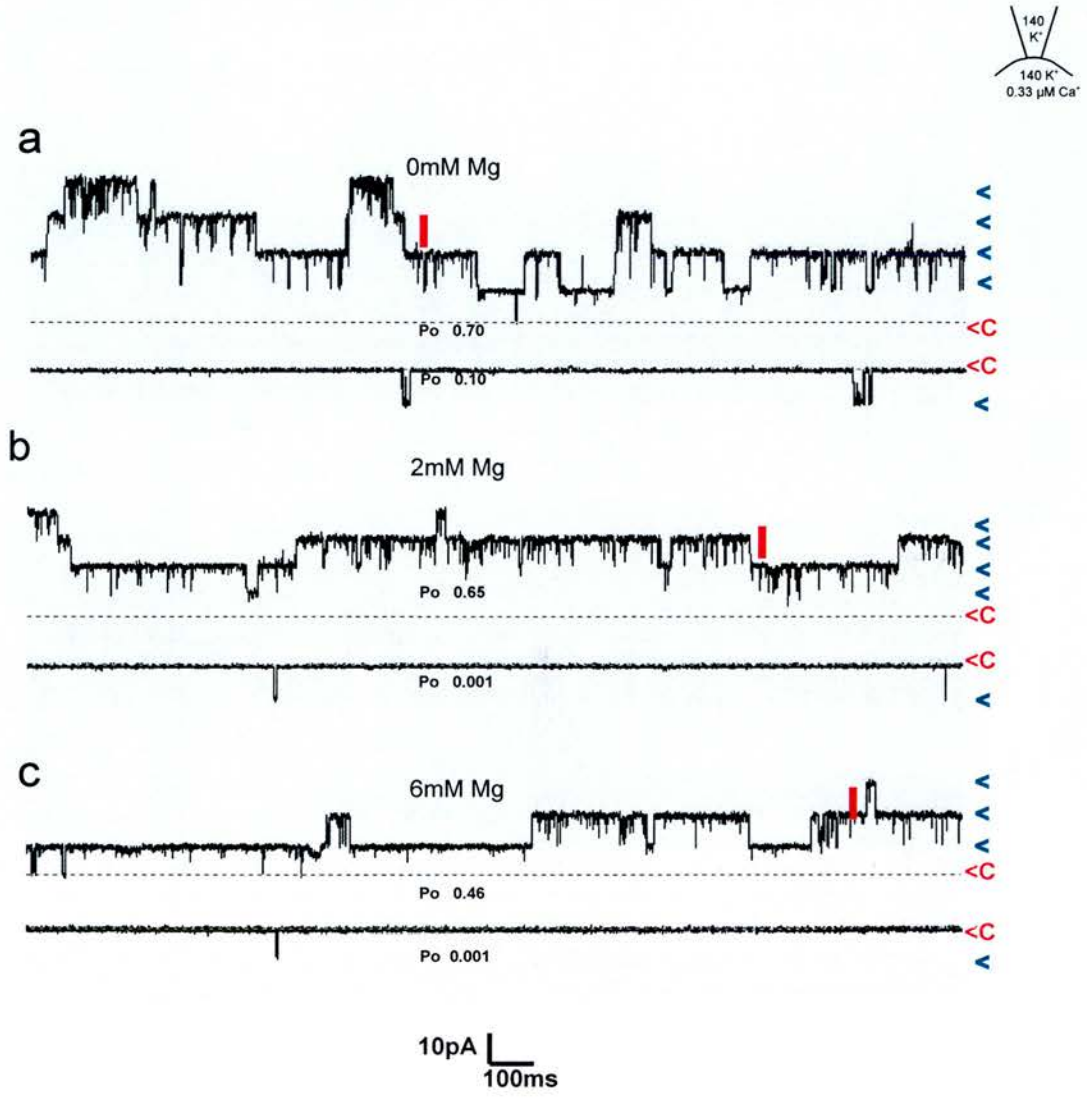
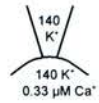


Figure 5.24 In excised inside out patches of HEK-293 cells transiently expressing STREX-E374A, increasing the concentration of magnesium at the intracellular face reduced the single channel amplitude Representative single channel traces at +/- 40mV in 0.33µM [Ca2+] are shown (panel a is 0mM magnesium, b is 2mM magnesium and c is illustrating the response in 6mM magnesium and b with the addition of lithium, C indicates closed state and > showing open states). Symbol ▮ indicates 10pA on the traces, to compare the change in the single channel amplitude upon addition of increasing magnesium. The Po values are shown on the traces and display a significant increase in channel activity as the concentration of magnesium is increased.

Figure 5.25
Magnesium reduces single channel amplitude of STREX-E374A channel



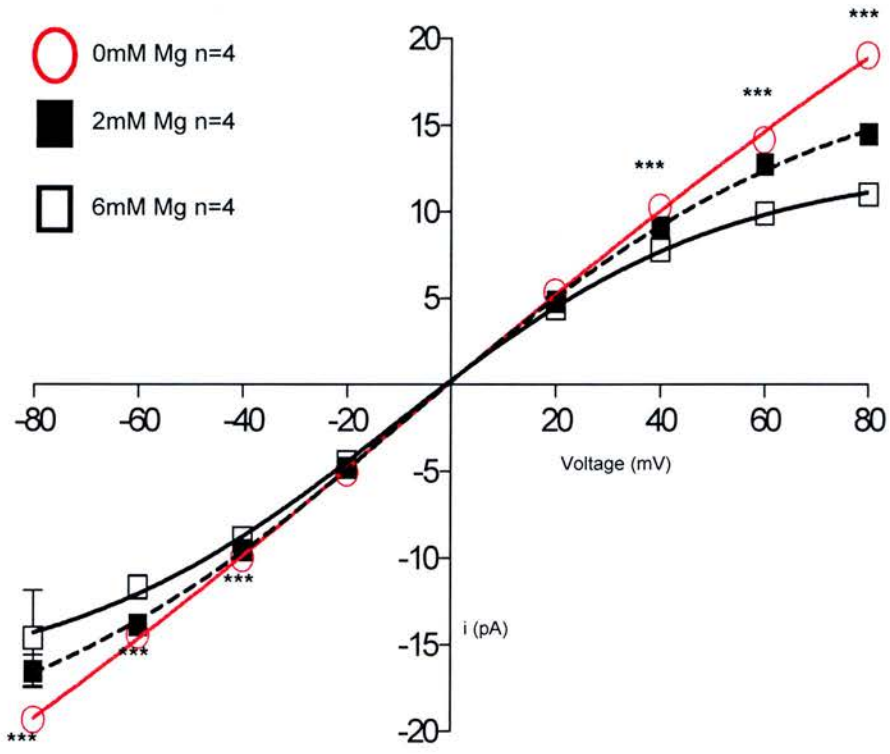


Figure 5.25 Site-directed mutagenesis at the E374 site did not alter the decrease in single channel amplitude caused by magnesium at positive potentials. However a decrease in channel amplitude by magnesium was observed at negative potentials in the STREX-E374A mutant (Data are mean ± s.e.m, *** $p \leq 0.001$ compared to other groups, ANOVA plus Tukey posthoc).

Figure 5.26
Magnesium and lithium alter channel activation

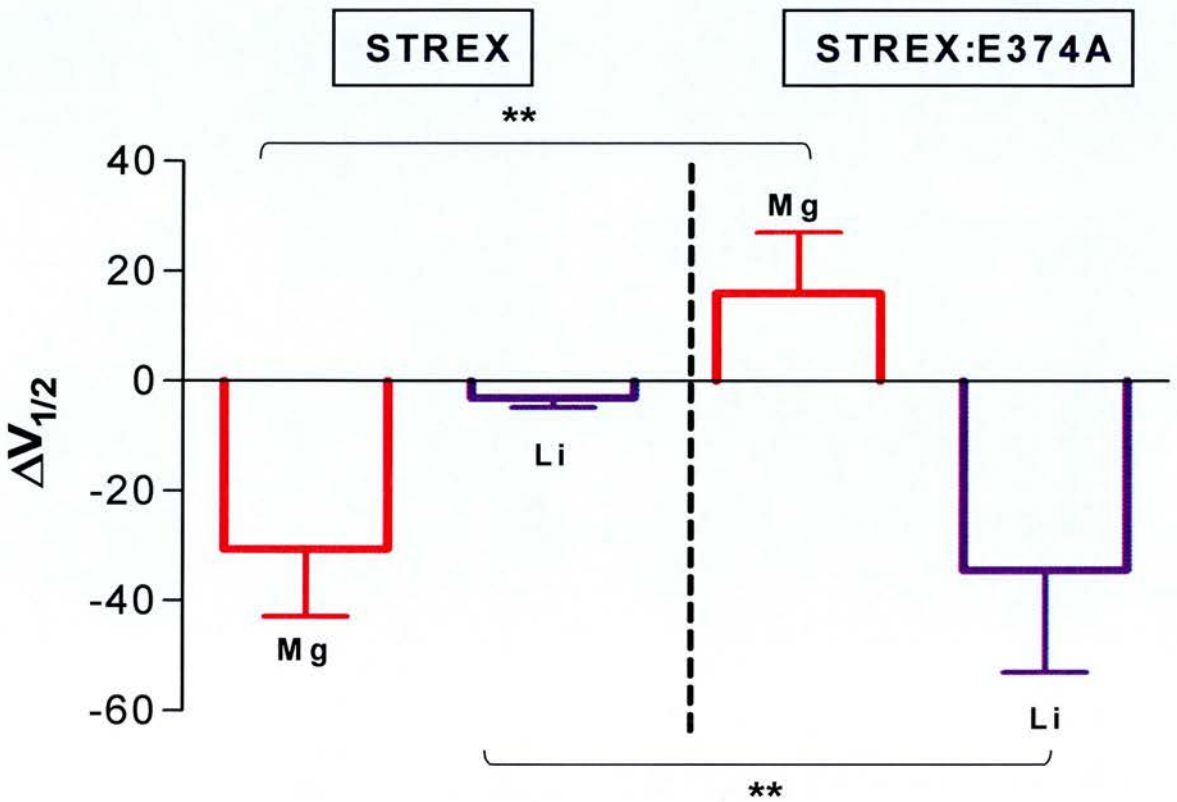


Figure 5.26 Increasing the magnesium concentration causes a significant rise in STREX activity as observed in the leftward shift of $V_{1/2}$, which was calculated using the Boltzmann equation to fit the activation curves. Lithium had no effect on STREX $V_{1/2}$.

In contrast, magnesium decreased activity in STREX:E374A producing a rightward shift in $V_{1/2}$. Additionally, lithium now produced a significant increase in the activity of the mutant STREX:E374A. (Data are mean ± s.e.m n=4, ** $p<0.01$ students Paired t-test).

5.3 Summary

The two main conclusions from the data presented in this section are: that the response of STREX channels to hypoxia can be modulated by the presence of intracellular ATP, and that lithium consistently and robustly blocks the hypoxia mediated inhibition of STREX channels.

5.3.1 ATP modulates the response of STREX channels to hypoxia

The application of intracellular ATP (in the presence of magnesium hence magnesium-ATP) to STREX channels under conditions of low oxygen tension produced two populations of responses. One group that was not significantly different to the control STREX channels response to hypoxia and one that caused channel activation in response to hypoxia. This implies that there is a patch to patch difference, and rather than the effect being directly on the channel itself there may be an ATP-sensitive protein which is present in only 50% of patches excised. It could also be that the channel is required to be in a certain state for the activation response to be elicited e.g. phosphorylated state.

Therefore ATP-dependent modulation of the channel may rely on the prior state of the channel in the patch.

So is the ATP effect a phosphorylation dependent mechanism or a phosphorylation independent mechanism? The pharmacological investigation of this effect would be complicated hence I used a direct site-directed mutagenesis approach to mutate the RxCS motif as it is a potential phosphorylation motif. This revealed that mutation of the R21 and S24 residues to alanine abolished the response of STREX channels to hypoxia and that the phosphomimetic mutant S24E also prevented the inhibition of STREX channel activity under conditions of low oxygen tension. Thus assuming that S24E was in fact a good phosphomimetic, this suggests that the lack of response of STREX channels to hypoxia is more to do with structural changes or modification of the local protein environment. Therefore implying that phosphorylation of the RxCS motif does not contribute to the ATP dependent modulation of the STREX channels response to hypoxia.

To address if phosphorylation globally may have an effect on channel activity under hypoxic conditions, I conducted experiments in the presence of

intracellular ATP but without magnesium and also using the poorly hydrolysable analogue of ATP AMP-PNP. Data obtained from these experiments was similar to the ATP data, suggesting that the mechanism of ATP modulation of STREX channels response to hypoxia is likely to be phosphorylation independent. This could be tested further by using other non-hydrolysable analogues of ATP. Commercially available analogues are either magnesium or lithium salts, and as shown here lithium itself abolishes the STREX channels response to hypoxia. Thus generation of non-hydrolysable analogues of ATP, for example a sodium salt would be invaluable for these studies. This would help to determine if the ATP modulation is via a phosphorylation dependent or independent mechanism, as for example investigated for K_{ATP} channel (Nichols, 2006).

5.3.2 Lithium blocks the response of STREX channels to hypoxia

Control experiments administering intracellular lithium surprisingly blocked the response of STREX channels to low oxygen tension, in a robust and consistent fashion. As lithium and magnesium share physicochemical properties, the effect of additional magnesium at the intracellular face of the

channel was also investigated. However magnesium did not abolish the response of STREX channels to hypoxia, therefore the mechanism of lithium-mediated block of STREX channels response to hypoxia must be different to that of magnesium. Nonetheless both cations had similar effects on reducing single channel amplitude of STREX and ZERO channels. These effects on single channel conductance were independent of the STREX insert and of the previously identified high-affinity magnesium binding site E374 (Yang *et al.*, 2006).

Therefore, could the decrease in single channel amplitude induced by intracellular lithium be mediated via the same mechanism that abolishes the response of STREX channels to hypoxia? It is known that there is a "ring of charge" at the entrance of the inner vestibule of the BK channel (Brelidze *et al.*, 2003) conferred by the presence of eight negatively charged glutamate residues. Recent data has also suggested that the ring of negative charge facilitates voltage-dependent blockade of BK channels by intracellular magnesium ions (Zhang *et al.*, 2006), thus it is reasonable to hypothesize that lithium could have a similar effect on BK channels. So could it be that the decrease in single channel amplitude conferred by lithium contributes to its block of STREX channels

response to hypoxia, in that the state of the channel is changed by lithium? This could be investigated by mutating the eight glutamate residues which confer this "ring of charge" and investigating what effect this has on a) the effect of lithium on single channel amplitude, and b) the lithium-mediated block of the response of STREX channels to hypoxia.

If these experiments revealed that the lithium block of the response of STREX channels to hypoxia was independent of effects on channel conductance, alternative mechanisms of action would need to be explored. Lithium regulates many other signalling pathways involving lipid and protein kinases, including glycogen synthase kinase 3, protein kinase C and inositol polyphosphate 1-phosphatase (Gould *et al.*, 2004). Thus lithium may regulate other signalling pathways to regulate the response of STREX channels to hypoxia, assuming that these proteins form part of the BK scaffold at the membrane or are co-localised near the BK channel at the membrane. Specific pharmacological tools targeting these kinases could be utilised to investigate the mechanism of the lithium induced block of the response of STREX channels to hypoxia.

This section therefore shows that the response of STREX channels to hypoxia isn't fixed but open to modulation, which may be dependent on the prior status of the channel protein. Investigating the mode of action of this could open up the physiological relevance of the response of STREX channels to hypoxia.

Chapter Six: Summary and conclusions

This thesis had three primary aims:

1) To develop a high throughput assay to allow discrimination of different BK channel splice variants and their modulation
2) To characterise the role of cysteine residues within the STREX insert that may determine hypoxia sensitivity of the channel
3) To examine the modulation of the hypoxia response by intracellular ATP

I developed a high-throughput screening assay exploiting voltage sensitive fluorescent dyes to discriminate between BK channel splice variants. This assay was used to address the functional impact of BK channel mutants and to probe the differential pharmacology between the splice variants. Secondly, I identified cysteine residues 23 (C23) as the key amino acid residue within the STREX insert required for the hypoxia mediated regulation of the STREX channel, which did not functionally interact with any other cysteine residues within the STREX insert to control sensitivity to hypoxia. My third finding was that intracellular ATP modulated the response of STREX channels to hypoxia; this could be via an ATP-dependent but phosphorylation independent mechanism. Finally, the monovalent lithium ion prevented the hypoxia mediated regulation of STREX channels.

6.1 Development of FMP assay for BK channel splice variants

The voltage-sensitive fluorescent membrane potential (FMP) assay was successfully developed as a preliminary high-throughput screen to investigate BK channel splice variants, utilising the calcium ionophore ionomycin to allow calcium influx to activate the channel. The assay allowed discrimination between distinct BK channel splice variants, based on their different calcium sensitivities (STREX > e22 > ZERO) and was proof of principal that this assay is viable for preliminary screening. It allowed investigation of different cysteine mutants of the STREX BK channel variant leading to the identification of the C12 and C13 as key residues that potentially contribute to the enhanced apparent calcium sensitivity of STREX channels. Furthermore the assay revealed distinct pharmacological profiles of the different variants to previously identified BK channel inhibitors. Thus the assay could potentially be used to screen compound libraries for novel modulators of specific BK splice variants, although drugs identified from these screens would still require verification via electrophysiology assays as it is still the "gold standard" for ion channel drug discovery.

6.1.1 Contribution of cysteine residues to apparent calcium sensitivity of STREX channels

The FMP assay highlighted the C12 and C13 as key cysteine residues that contribute to the enhanced apparent calcium sensitivity of the STREX channel. However, mutation of the C23 and C25 residues also affects the baseline open probability of the channel in electrophysiological assays (Section 4.2) but do not show significant changes of the ionomycin-induced hyperpolarisation in the FMP assay (Sections 3.2.3). As discussed in Section 4.3, the difference could be attributed to the calcium and voltage driven responses. Thus it would be necessary to isolate the calcium response from the voltage response using patch clamp electrophysiology to determine which residues are indeed vital for the enhanced apparent calcium sensitivity of STREX channels. The structural implications on the STREX channel of the residues identified could reveal potential interactions between the cysteine residues. This could be via disulphide bonds outwith the STREX insert on the same α subunit thus intra-subunit interactions, or indeed mediating inter-subunit interactions, thereby altering the conformation of the channel to provide it with this increased apparent calcium sensitivity.

6.1.2 Differential pharmacological profile of BK channel splice variants

The FMP assay allowed the identification of specific BK channel inhibitors with increased potency on the three variants STREX, e22 and ZERO, thus opening their potential use as probes used to distinguish between different BK channel variants expressed in native systems. Of the indole diterpene alkaloids verruculogen displayed a significantly greater potency (4 fold order of magnitude) on the ZERO and e22 variants compared to STREX channels. In contrast e22 channels were significantly less potently inhibited by paxilline, whereas STREX channels were more sensitive to penitrem A.

In the absence of a 3D structure of BK channels, it is difficult to conclude why such distinct differences in potency are apparent between the indole diterpene alkaloid inhibitors on the three BK channel variants. However it can be hypothesized that perhaps the different length of the inserted exons, or lack thereof, alters the folding of the large C-terminus end of the channel, which perhaps changes it's interaction with the channel pore. These differences in inhibitor potency would require further investigation using patch clamp

electrophysiology to observe single-channel kinetics and changes in channel activity to provide more information on the mode of action of this class of drugs.

Unexpectedly the classic BK inhibitors, TEA and iberiotoxin, that are commonly used in the lab as pharmacological tools, did not elicit full inhibition of the ionomycin-induced channel hyperpolarisation in this assay. These two compounds are thought to block BK channel activity by directly blocking the pore of the channel. TEA mediates a "flickery" block of the channel pore, whilst iberiotoxin "sits" in the channel pore to prolong the channel closed times. In contrast, the indole diterpenes are thought to induce an allosteric conformational change of the channel pore rather than acting as conventional pore blockers.

Therefore it can be hypothesized that in the FMP assay these two different mechanisms of action could be responsible for the difference in potency observed. The ionomycin stimulated influx of calcium ions into a population of cells may result in sufficient ion conductance through the TEA and iberiotoxin "block" of BK channels, that enough whole cell current is present to elicit significant changes in membrane potential, as reported by the FMP assay. However the FMP dye emits fluorescence by binding to cytosolic proteins whilst

any extracellular dye is quenched, therefore we cannot completely eliminate drug interference with the dye itself or the quencher, which could falsely be reporting the poor potency of TEA and iberiotoxin in the FMP assay.

6.1.3 Future use of the developed FMP assay

Previous physiological and pharmacological characterisation of BK channel modulators has depended on using low-throughput methods such as ligand binding or electrophysiology analysis. The technical ease of the FMP assay renders it an excellent high-throughput preliminary screen for investigating functional BK channel properties. For example, it's interactions with other proteins thought to be present within the channel "scaffold" at the membrane, and expression in native cell lines. This would allow the identification of drugs and channel-associated proteins that may interact with the BK channel and its splice variants. The validation of any interesting effects thus identified would require confirmation using the ion channel "gold standard", electrophysiology. These experiments could actually be conducted in parallel using new medium-throughput ion channel screening methods, for example the Port-a-Patch (Fertig *et al.*, 2002; Fertig *et al.*, 2003) and IonWorks^{HT} (Dale *et al.*, 2007; John *et al.*, 2007).

Thus the development of the high-throughput FMP assay allows faster resolution to addressing fundamental questions in the ion channel field.

6.2 Role of cysteine residues in the hypoxia sensitivity of STREX BK channels

BK channel activity is affected by acute and chronic changes in oxygen tension; however the mechanism of action behind this effect is controversial (Kemp, 2006). Is it a direct consequence of reduced oxygen tension on the channel (i.e. the mechanism is membrane delimited) or are cytosolic factors required to mediate the change in BK channel activity? Currently the three key proposed mechanisms implicated in the hypoxic inhibition of BK channels are: a) Hemoxygenase-2 (Williams *et al.*, 2004), b) AMP kinase (Evans *et al.*, 2005; Wyatt *et al.*, 2007) and c) STREX splice variant selectivity (McCartney *et al.*, 2005).

The data presented in this thesis shows that acute hypoxia inhibits recombinant BK channel activity (70-80%) in excised membrane patches, via a rapid and reversible mechanism that is specific to the pore-forming α subunits of the

cysteine-rich STREX BK channel variant. Previously an evolutionary conserved hypoxia sensing motif (-CSC-) was identified in the cysteine-rich STREX insert (McCartney *et al.*, 2005). It was proposed therefore that this change in STREX channel activity under low oxygen tensions might be due to the disruption of disulphide bonds thereby changing the conformation of the channel to decrease channel activity. Here a site-directed mutagenesis strategy combined with single channel patch clamp recording in excised inside-out patches was used to examine the contribution of all cysteine residues within the STREX insert on hypoxia sensitivity. Of all the cysteine residues (C12, C13, C16, C23, C25 and C51) in the STREX insert; mutation of the C23 site alone completely abolished the response to hypoxia.

As discussed in Section 4.3, the C23 residue could be responsible for inducing structural changes within the channel in response to hypoxia by mediating intra- or inter-subunit interactions (Figure 6.1, Model A and C). BK channels are tetramers of four pore-forming α subunits. It is therefore possible that the single mutation of the C23 residue prevents inter-subunit interactions such as disulphide bond formation between STREX variants in different α subunits (Figure 6.1, Model A), as well as interactions of C23 with other cysteine residues

in neighbouring α subunits (Figure 6.1, Model B). As proposed in Section 4 TEA insensitive subunits could be utilised to investigate if inter-subunit interactions have a role in the STREX hypoxic response. Channel homomers and heteromers of the STREX-C23A mutant and STREX wildtype channel could be constructed to determine if inter-subunit interactions mediated by the C23 residue are altered during hypoxic exposure and could be responsible for the change in BK channel activity under conditions of low oxygen tension.

The lack of any crystallisation of STREX at present to elucidate the 3D structure means that to investigate if the STREX hypoxia response is mediated by a change in the functional conformation of the channel it would be necessary to employ novel fluorescence lifetime imaging microscopy (FLIM)-based FRET assays. FLIM can be used as an accurate and quantitative assay to study conformational changes of proteins in cells (Duncan *et al.*, 2004). By using fluorescent fusion proteins tagged onto the functional STREX channel or indeed truncated C-terminus segments crucial insights into the structural changes that occur in STREX during exposure to hypoxic conditions could be gained.

Figure 6.1
Models of potential C23 interactions

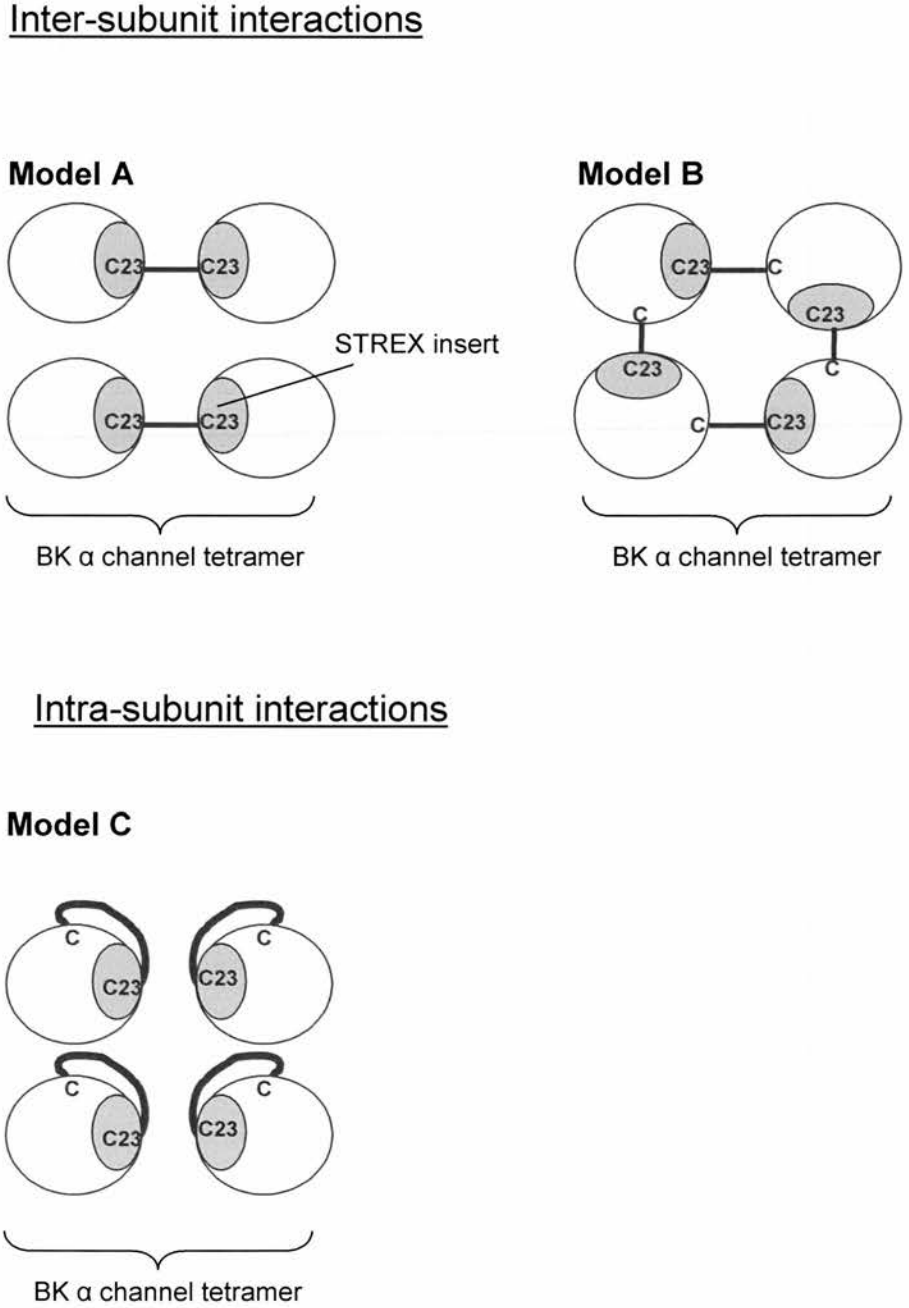


Figure 6.1 Diagrammatic representation of BK α subunit tetramers, with the STREX insert indicated within each individual α subunit in light blue. C23 residue indicated, with C representing other cysteine residues in the channel. Models of potential inter- and intra-subunit interactions are indicated which may be responsible for the response of STREX channels to hypoxia.

6.3 Modulation of the response of STREX channels to hypoxia by intracellular ATP and lithium

6.3.1 Modulation of the response of STREX channels to hypoxia by intracellular ATP

Intracellular ATP revealed a population of STREX channels that responded with an increase in activity in response to hypoxia, in addition to the normal hypoxia mediated inhibition of STREX channels. The ATP dependent activation of STREX channels by hypoxia could potentially be a physiological control mediated by the cell, in that under conditions of high intracellular ATP concentration the cells metabolism is not yet compromised and BK channel activation can thereby allow cell hyperpolarisation so overriding the protective hypoxic response? As the cysteine rich hypoxia sensing motif in STREX lies within a putative phosphorylation motif ($RxCS/R_{21}XC_{23}S_{24}$) in an attempt to identify if the ATP modulation was via a phosphorylation dependent or independent mechanism site-directed mutagenesis of the RxCS site was carried out. Mutation of the key R and S amino acid residues in the motif to alanine prevented the hypoxic inhibitory response, suggesting that the intact RxCS site

in STREX is required for the response to hypoxia. Interestingly, neither the phospho-null (STREX-S24A) nor phosphomimetic (STREX-S24E) mutant showed any significant changes in channel behaviour under hypoxia which were indistinguishable from one another, suggesting that phosphorylation at this site does not mediate the action of ATP and that the effects of mutation are a result of fundamental structural changes.

In the presence of a non-hydrolysable analogue of ATP, AMP-PNP (a tetra-lithium salt), the response of STREX channels to hypoxia was similar to that observed with ATP. This provides further evidence that ATP may mediate STREX channel activation under hypoxic conditions via a phosphorylation independent mechanism. The concentration of cellular ATP changes under hypoxic conditions (Section 5.1.1), thus it would be useful to utilise other non-hydrolysable analogues of ATP to decipher ATP modulation of STREX channel activity to reveal its physiological role.

6.3.2 Blockade of the response of STREX channels to hypoxia by intracellular lithium ions

During the course of these experiments the monovalent ion lithium was observed to robustly block the response of STREX channels to hypoxia. As lithium shares physicochemical properties with the divalent magnesium ion, the role of magnesium in the STREX hypoxia response was also investigated. Magnesium appeared to modulate the response of STREX channels to hypoxia in a similar fashion to ATP, in that it was variable and distinctly different to the robust inhibition seen in the presence of intracellular lithium. In addition it was seen that lithium and magnesium decreased the single channel conductance of STREX and ZERO BK channel variants, magnesium also increased STREX channel open probability. Therefore it could be possible that the changes in channel conductance induced by lithium may contribute to its inhibition of the response of STREX channels to hypoxia. Alternatively as lithium affects other signalling pathways, there are other mechanisms by which lithium may work.

6.4 Implications for understanding the structure/ function relationship of the BK channel

Taken together, these data suggest that alternative pre-mRNA splicing is an important determinant of BK channel pharmacology, and that cysteine and other residues within the STREX insert play a key role in the function of the channel and its response to hypoxia. This implies that the splice variants induce changes in the structure of the channel that affect both the conformation of the intracellular C-terminus as well as the extracellular vestibule of the pore.

As the STREX insert provides the channel with enhanced calcium sensitivity, how could this insert significantly be changing the structure of the pore region and affecting channel activity, gating and pharmacology? If we look at the structure of the BK channel α subunit (Figure 1.2), we see that the STREX insert would be predicted to lie in between the two proposed RCK domains and upstream of the proposed calcium bowl containing RCK2 domain. Recent work has shown that binding of calcium to the C-terminus of the BK channel and the interaction of these two RCK domains via a fixed or flexible interface (Schumacher & Adelman, 2002; Qian *et al.*, 2006) has an important impact on the

opening of the channel pore as well as affecting the selectivity filter. With the RCK2 domain now proposed as a calcium bowl region and potential cation binding sites (M513, D367, D362, E374, Q397 and E399) present in the RCK1 domain also (Yusifov *et al.*, 2008), it is reasonable to propose that the enhanced calcium and hypoxia sensitivity provided by the STREX insert is in fact due to interactions it mediates with these domains thus directly impacting the gating of the channel. Accordingly, if hypoxia causes a change in the structural configuration of the STREX insert, this may ultimately affect the interaction between the RCK1 and RCK2 domains thus inducing the decrease in the STREX channel activity. Lithium, magnesium and ATP could also in someway interact with the RCK1 and RCK2 structures, as well as the "ring of charge". This may potentially modify the overall structural configuration of the C-terminus and control the ability of hypoxia to induce a decrease in channel activity by mediating structural changes in the STREX channel.

Consequently, BK channel splice variants could induce changes in C-terminus interactions in the BK channel that may potentially affect the external vestibule of the pore. This may alter the binding of BK channel inhibitors by presenting different pharmacologically active sites, explaining the differential sensitivity of

the conformational inhibitors the indole diterpenes, and the pore blocking TEA and iberiotoxin. Therefore potential BK channel specific drugs should be screened on all the alternatively spliced variants of the BK channel, not just those at the c2 site but all the other C and N terminus splice variants, as they could potentially present very different pharmacological behaviour. Crystallisation of STREX would provide a crucial insight to the structural changes that are induced by hypoxia and possibly a pharmacaphore which mediates differential effects of the BK specific inhibitors.

References:

Adachi T, Ishikawa K, Hida W, Matsumoto H, Masuda T, Date F, Ogawa K, Takeda K, Furuyama K, Zhang Y, Kitamuro T, Ogawa H, Maruyama Y & Shibahara S. (2004). Hypoxemia and blunted hypoxic ventilatory responses in mice lacking heme oxygenase-2. *Biochem Biophys Res Commun* **320,** 514-522.

Adelman JP, Shen KZ, Kavanaugh MP, Warren RA, Wu YN, Lagrutta A, Bond CT & North RA. (1992). Calcium-activated potassium channels expressed from cloned complementary DNAs. *Neuron* **9,** 209-216.

Ahluwalia J, Tinker A, Clapp LH, Duchen MR, Abramov AY, Pope S, Nobles M & Segal AW. (2004). The large-conductance Ca2+-activated K+ channel is essential for innate immunity. *Nature* **427,** 853-858.

Aitken A. (1999). Protein consensus sequence motifs. *Mol Biotechnol* **12,** 241-253.

Alioua A, Mahajan A, Nishimaru K, Zarei MM, Stefani E & Toro L. (2002). Coupling of c-Src to large conductance voltage- and Ca2+-activated K^+ channels as a new mechanism of agonist-induced vasoconstriction. *Proc Natl Acad Sci U S A* **99,** 14560-14565.

Amberg GC & Santana LF. (2003). Downregulation of the BK channel β1 subunit in genetic hypertension. *Circ Res* **93,** 965-971.

Anderson JA, Nakamura RL & Gaber RF. (1994). Heterologous expression of K^+ channels in Saccharomyces cerevisiae: strategies for molecular analysis of structure and function. *Symp Soc Exp Biol* **48,** 85-97.

Apell HJ & Bersch B. (1987). Oxonol VI as an optical indicator for membrane potentials in lipid vesicles. *Biochim Biophys Acta* **903,** 480-494.

Archer SL, London B, Hampl V, Wu X, Nsair A, Puttagunta L, Hashimoto K, Waite RE & Michelakis ED. (2001). Impairment of hypoxic pulmonary

vasoconstriction in mice lacking the voltage-gated potassium channel Kv1.5. *Faseb J* **15,** 1801-1803.

Atkinson NS, Robertson GA & Ganetzky B. (1991). A component of calcium-activated potassium channels encoded by the Drosophila slo locus. *Science* **253,** 551-555.

Babot Z, Cristofol R & Sunol C. (2005). Excitotoxic death induced by released glutamate in depolarized primary cultures of mouse cerebellar granule cells is dependent on GABAA receptors and niflumic acid-sensitive chloride channels. *Eur J Neurosci* **21,** 103-112.

Bahring R, Dannenberg J, Peters HC, Leicher T, Pongs O & Isbrandt D. (2001). Conserved Kv4 N-terminal domain critical for effects of Kv channel-interacting protein 2.2 on channel expression and gating. *J Biol Chem* **276,** 23888-23894.

Bao L & Cox DH. (2005). Gating and ionic currents reveal how the BK_{Ca} channel's Ca^{2+} sensitivity is enhanced by its beta1 subunit. *J Gen Physiol* **126,** 393-412.

Bao L, Rapin AM, Holmstrand EC & Cox DH. (2002). Elimination of the $BK_{(Ca)}$ channel's high-affinity $Ca_{(2+)}$ sensitivity. *J Gen Physiol* **120,** 173-189.

Barman SA, Zhu S, Han G & White RE. (2003). cAMP activates BK_{Ca} channels in pulmonary arterial smooth muscle via cGMP-dependent protein kinase. *Am J Physiol Lung Cell Mol Physiol* **284,** L1004-1011.

Barman SA, Zhu S & White RE. (2004). PKC activates BK_{Ca} channels in rat pulmonary arterial smooth muscle via cGMP-dependent protein kinase. *Am J Physiol Lung Cell Mol Physiol* **286,** L1275-1281.

Baxter DF, Kirk M, Garcia AF, Raimondi A, Holmqvist MH, Flint KK, Bojanic D, Distefano PS, Curtis R & Xie Y. (2002). A novel membrane potential-sensitive fluorescent dye improves cell-based assays for ion channels. *J Biomol Screen* **7,** 79-85.

Behrens R, Nolting A, Reimann F, Schwarz M, Waldschutz R & Pongs O. (2000). hKCNMB3 and hKCNMB4, cloning and characterization of two members of the large-conductance calcium-activated potassium channel beta subunit family. *FEBS Lett* **474,** 99-106.

Benot AR & Lopez-Barneo J. (1990). Feedback Inhibition of Ca^{2+} Currents by Dopamine in Glomus Cells of the Carotid Body. *Eur J Neurosci* **2,** 809-812.

Biagi G, Calderone V, Giorgi I, Livi O, Martinotti E, Martelli A & Nardi A. (2004a). 1,5-Diarylsubstituted 1,2,3-triazoles as potassium channel activators. VI. *Farmaco* **59,** 397-404.

Biagi G, Giorgi I, Livi O, Nardi A, Calderone V, Martelli A, Martinotti E & LeRoy Salerni O. (2004b). Synthesis and biological activity of novel substituted benzanilides as potassium channel activators. V. *Eur J Med Chem* **39,** 491-498.

Bian S, Favre I & Moczydlowski E. (2001). Ca^{2+}-binding activity of a COOH-terminal fragment of the Drosophila BK channel involved in Ca^{2+}-dependent activation. *Proc Natl Acad Sci U S A* **98,** 4776-4781.

Bielefeldt K, Rotter JL & Jackson MB. (1992). Three potassium channels in rat posterior pituitary nerve terminals. *J Physiol* **458,** 41-67.

Boris-Moller F & Wieloch T. (1998). Changes in the extracellular levels of glutamate and aspartate during ischemia and hypoglycemia. Effects of hypothermia. *Exp Brain Res* **121,** 277-284.

Brayden JE & Nelson MT. (1992). Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science* **256,** 532-535.

Brelidze TI, Niu X & Magleby KL. (2003). A ring of eight conserved negatively charged amino acids doubles the conductance of BK channels and prevents inward rectification. *Proc Natl Acad Sci U S A* **100,** 9017-9022.

Brenner R, Chen QH, Vilaythong A, Toney GM, Noebels JL & Aldrich RW. (2005). BK channel β4 subunit reduces dentate gyrus excitability and protects against temporal lobe seizures. *Nat Neurosci* **8,** 1752-1759.

Brenner R, Jegla TJ, Wickenden A, Liu Y & Aldrich RW. (2000a). Cloning and functional characterization of novel large conductance calcium-activated potassium channel beta subunits, hKCNMB3 and hKCNMB4. *J Biol Chem* **275,** 6453-6461.

Brenner R, Perez GJ, Bonev AD, Eckman DM, Kosek JC, Wiler SW, Patterson AJ, Nelson MT & Aldrich RW. (2000b). Vasoregulation by the β1 subunit of the calcium-activated potassium channel. *Nature* **407,** 870-876.

Bringmann A, Faude F & Reichenbach A. (1997). Mammalian retinal glial (Muller) cells express large-conductance $Ca(^{2+})$-activated K^+ channels that are modulated by Mg^{2+} and pH and activated by protein kinase A. *Glia* **19,** 311-323.

Brodie MS, Scholz A, Weiger TM & Dopico AM. (2007). Ethanol interactions with calcium-dependent potassium channels. *Alcohol Clin Exp Res* **31,** 1625-1632.

Buckler KJ. (1997). A novel oxygen-sensitive potassium current in rat carotid body type I cells. *J Physiol* **498 (Pt 3),** 649-662.

Buckler KJ & Honore E. (2005). The lipid-activated two-pore domain K+ channel TREK-1 is resistant to hypoxia: implication for ischaemic neuroprotection. *J Physiol* **562,** 213-222.

Buckler KJ, Williams BA & Honore E. (2000). An oxygen-, acid- and anaesthetic-sensitive TASK-like background potassium channel in rat arterial chemoreceptor cells. *J Physiol* **525 Pt 1,** 135-142.

Bunn HF & Poyton RO. (1996). Oxygen sensing and molecular adaptation to hypoxia. *Physiol Rev* **76,** 839-885.

Burdyga T & Wray S. (2005). Action potential refractory period in ureter smooth muscle is set by Ca sparks and BK channels. *Nature* **436,** 559-562.

Butler A, Tsunoda S, McCobb DP, Wei A & Salkoff L. (1993). mSlo, a complex mouse gene encoding "maxi" calcium-activated potassium channels. *Science* **261,** 221-224.

Campanucci VA, Brown ST, Hudasek K, O'Kelly I M, Nurse CA & Fearon IM. (2005). O2 sensing by recombinant TWIK-related halothane-inhibitable K+ channel-1 background K+ channels heterologously expressed in human embryonic kidney cells. *Neuroscience* **135,** 1087-1094.

Candia S, Garcia ML & Latorre R. (1992). Mode of action of iberiotoxin, a potent blocker of the large conductance $Ca^{(2+)}$-activated K^+ channel. *Biophys J* **63,** 583-590.

Catterall WA. (1995). Structure and function of voltage-gated ion channels. *Annu Rev Biochem* **64,** 493-531.

Chen L, Tian L, MacDonald SH, McClafferty H, Hammond MS, Huibant JM, Ruth P, Knaus HG & Shipston MJ. (2005). Functionally diverse complement of large conductance calcium- and voltage-activated potassium channel (BK) α-subunits generated from a single site of splicing. *J Biol Chem* **280,** 33599-33609.

Chung SK, Reinhart PH, Martin BL, Brautigan D & Levitan IB. (1991). Protein kinase activity closely associated with a reconstituted calcium-activated potassium channel. *Science* **253,** 560-562.

Coppock EA & Tamkun MM. (2001). Differential expression of K(V) channel alpha- and beta-subunits in the bovine pulmonary arterial circulation. *Am J Physiol Lung Cell Mol Physiol* **281,** L1350-1360.

Dale TJ, Townsend C, Hollands EC & Trezise DJ. (2007). Population patch clamp electrophysiology: a breakthrough technology for ion channel screening. *Mol Biosyst* **3,** 714-722.

Darman RB, Flemmer A & Forbush B. (2001). Modulation of ion transport by direct targeting of protein phosphatase type 1 to the Na-K-Cl cotransporter. *J Biol Chem* **276,** 34359-34362.

Darman RB & Forbush B. (2002). A regulatory locus of phosphorylation in the N terminus of the Na-K-Cl cotransporter, NKCC1. *J Biol Chem* **277,** 37542-37550.

de-Allie FA, Bolsover SR, Nowicky AV & Strong PN. (1996). Characterization of Ca(2+)-activated 86Rb$^+$ fluxes in rat C6 glioma cells: a system for identifying novel IKCa-channel toxins. *Br J Pharmacol* **117,** 479-487.

Del Toro R, Levitsky KL, Lopez-Barneo J & Chiara MD. (2003). Induction of T-type calcium channel gene expression by chronic hypoxia. *J Biol Chem* **278,** 22316-22324.

Dong L, Zheng YM, Van Riper D, Rathore R, Liu QH, Singer HA & Wang YX. (2008). Functional and molecular evidence for impairment of calcium-activated potassium channels in type-1 diabetic cerebral artery smooth muscle cells. *J Cereb Blood Flow Metab* **28,** 377-386.

Doyle DA, Morais Cabral J, Pfuetzner RA, Kuo A, Gulbis JM, Cohen SL, Chait BT & MacKinnon R. (1998). The structure of the potassium channel: molecular basis of K+ conduction and selectivity. *Science* **280,** 69-77.

Du W, Bautista JF, Yang H, Diez-Sampedro A, You SA, Wang L, Kotagal P, Luders HO, Shi J, Cui J, Richerson GB & Wang QK. (2005). Calcium-sensitive potassium channelopathy in human epilepsy and paroxysmal movement disorder. *Nat Genet* **37,** 733-738.

Duncan RR, Bergmann A, Cousin MA, Apps DK & Shipston MJ. (2004). Multi-dimensional time-correlated single photon counting (TCSPC) fluorescence lifetime imaging microscopy (FLIM) to detect FRET in cells. *Journal of Microscopy-Oxford* **215,** 1-12.

Dunphy JT & Linder ME. (1998). Signalling functions of protein palmitoylation. *Biochim Biophys Acta* **1436,** 245-261.

Dworetzky SI, Trojnacki JT & Gribkoff VK. (1994). Cloning and expression of a human large-conductance calcium-activated potassium channel. *Brain Res Mol Brain Res* **27,** 189-193.

Epps DE, Wolfe ML & Groppi V. (1994). Characterization of the steady-state and dynamic fluorescence properties of the potential-sensitive dye bis-(1,3-dibutylbarbituric acid)trimethine oxonol (Dibac4(3)) in model systems and cells. *Chem Phys Lipids* **69,** 137-150.

Erdahl WL, Chapman CJ, Taylor RW & Pfeiffer DR. (2000). Ionomycin, a carboxylic acid ionophore, transports $Pb^{(2+)}$ with high selectivity. *J Biol Chem* **275,** 7071-7079.

Erxleben C, Everhart AL, Romeo C, Florance H, Bauer MB, Alcorta DA, Rossie S, Shipston MJ & Armstrong DL. (2002a). Interacting effects of N-terminal variation and strex exon splicing on slo potassium channel regulation by calcium, phosphorylation, and oxidation. *Journal of Biological Chemistry* **277,** 27045-27052.

Erxleben C, Everhart AL, Romeo C, Florance H, Bauer MB, Alcorta DA, Rossie S, Shipston MJ & Armstrong DL. (2002b). Interacting effects of N-terminal variation and strex exon splicing on slo potassium channel regulation by calcium, phosphorylation, and oxidation. *J Biol Chem* **277,** 27045-27052.

Evans AM, Mustard KJ, Wyatt CN, Peers C, Dipp M, Kumar P, Kinnear NP & Hardie DG. (2005). Does AMP-activated protein kinase couple inhibition of mitochondrial oxidative phosphorylation by hypoxia to calcium signaling in O2-sensing cells? *J Biol Chem* **280,** 41504-41511.

Farley J & Rudy B. (1988). Multiple types of voltage-dependent Ca^{2+}-activated K+ channels of large conductance in rat brain synaptosomal membranes. *Biophys J* **53,** 919-934.

Fearon IM, Campanucci VA, Brown ST, Hudasek K, O'Kelly IM & Nurse CA. (2006). Acute hypoxic regulation of recombinant THIK-1 stably expressed in HEK293 cells. *Adv Exp Med Biol* **580,** 203-208; discussion 351-209.

Fernandez-Fernandez JM, Tomas M, Vazquez E, Orio P, Latorre R, Senti M, Marrugat J & Valverde MA. (2004). Gain-of-function mutation in the KCNMB1 potassium channel subunit is associated with low prevalence of diastolic hypertension. *J Clin Invest* **113,** 1032-1039.

Fertig N, Blick RH & Behrends JC. (2002). Whole cell patch clamp recording performed on a planar glass chip. *Biophys J* **82,** 3056-3062.

Fertig N, George M, Klau M, Meyer C, Tilke A, Sobotta C, Blick RH & Behrends JC. (2003). Microstructured apertures in planar glass substrates for ion channel research. *Receptors Channels* **9,** 29-40.

Fettiplace R & Fuchs PA. (1999). Mechanisms of hair cell tuning. *Annu Rev Physiol* **61,** 809-834.

Flemmer AW, Gimenez I, Dowd BF, Darman RB & Forbush B. (2002). Activation of the Na-K-Cl otransporter NKCC1 detected with a phospho-specific antibody. *J Biol Chem* **277,** 37551-37558.

Fluhler E, Burnham VG & Loew LM. (1985). Spectra, membrane binding, and potentiometric responses of new charge shift probes. *Biochemistry* **24,** 5749-5755.

Fong N, Bird G, Vigneron M & Bentley DL. (2003). A 10 residue motif at the C-terminus of the RNA pol II CTD is required for transcription, splicing and 3' end processing. *Embo J* **22,** 4274-4282.

Galvez A, Gimenez-Gallego G, Reuben JP, Roy-Contancin L, Feigenbaum P, Kaczorowski GJ & Garcia ML. (1990). Purification and characterization of a unique, potent, peptidyl probe for the high conductance calcium-activated potassium channel from venom of the scorpion Buthus tamulus. *J Biol Chem* **265,** 11083-11090.

Ghatta S, Nimmagadda D, Xu X & O'Rourke ST. (2006). Large-conductance, calcium-activated potassium channels: structural and functional implications. *Pharmacol Ther* **110,** 103-116.

Ghezzi P & Bonetto V. (2003). Redox proteomics: identification of oxidatively modified proteins. *Proteomics* **3,** 1145-1153.

Giangiacomo KM, Garcia ML & McManus OB. (1992). Mechanism of iberiotoxin block of the large-conductance calcium-activated potassium channel from bovine aortic smooth muscle. *Biochemistry* **31,** 6719-6727.

Golowasch J, Kirkwood A & Miller C. (1986). Allosteric effects of Mg^{2+} on the gating of Ca^{2+}-activated K^+ channels from mammalian skeletal muscle. *J Exp Biol* **124,** 5-13.

Gonzalez JE, Oades K, Leychkis Y, Harootunian A & Negulescu PA. (1999). Cell-based assays and instrumentation for screening ion-channel targets. *Drug Discov Today* **4,** 431-439.

Gould TD, Quiroz JA, Singh J, Zarate CA & Manji HK. (2004). Emerging experimental therapeutics for bipolar disorder: insights from the molecular and cellular actions of current mood stabilizers. *Mol Psychiatry* **9,** 734-755.

Graumann J, Lilie H, Tang X, Tucker KA, Hoffmann JH, Vijayalakshmi J, Saper M, Bardwell JC & Jakob U. (2001). Activation of the redox-regulated molecular chaperone Hsp33--a two-step mechanism. *Structure* **9,** 377-387.

Gribkoff VK, Starrett JE, Jr. & Dworetzky SI. (2001a). Maxi-K potassium channels: form, function, and modulation of a class of endogenous regulators of intracellular calcium. *Neuroscientist* **7,** 166-177.

Gribkoff VK, Starrett JE, Jr., Dworetzky SI, Hewawasam P, Boissard CG, Cook DA, Frantz SW, Heman K, Hibbard JR, Huston K, Johnson G, Krishnan BS, Kinney GG, Lombardo LA, Meanwell NA, Molinoff PB, Myers RA, Moon SL, Ortiz A, Pajor L, Pieschl RL, Post-Munson DJ, Signor LJ, Srinivas N, Taber MT, Thalody G, Trojnacki JT, Wiener H, Yeleswaram K & Yeola SW. (2001b). Targeting acute ischemic stroke with a calcium-sensitive opener of maxi-K potassium channels. *Nat Med* **7,** 471-477.

Grunnet M & Kaufmann WA. (2004). Coassembly of big conductance Ca^{2+}-activated K^+ channels and L-type voltage-gated Ca^{2+} channels in rat brain. *J Biol Chem* **279,** 36445-36453.

Hagen BM, Bayguinov O & Sanders KM. (2003). β 1-subunits are required for regulation of coupling between Ca^{2+} transients and Ca^{2+}-activated K^+ (BK) channels by protein kinase C. *Am J Physiol Cell Physiol* **285,** C1270-1280.

Hamill OP, Marty A, Neher E, Sakmann B & Sigworth FJ. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch* **391,** 85-100.

Hampl V, Bibova J, Stranak Z, Wu X, Michelakis ED, Hashimoto K & Archer SL. (2002). Hypoxic fetoplacental vasoconstriction in humans is mediated by potassium channel inhibition. *Am J Physiol Heart Circ Physiol* **283,** H2440-2449.

Hanner M, Vianna-Jorge R, Kamassah A, Schmalhofer WA, Knaus HG, Kaczorowski GJ & Garcia ML. (1998). The beta subunit of the high conductance calcium-activated potassium channel. Identification of residues involved in charybdotoxin binding. *J Biol Chem* **273,** 16289-16296.

Hartness ME, Lewis A, Searle GJ, O'Kelly I, Peers C & Kemp PJ. (2001). Combined antisense and pharmacological approaches implicate hTASK as an airway O(2) sensing K(+) channel. *J Biol Chem* **276,** 26499-26508.

Haug T & Sand O. (1997). BK channels in intact clonal rat pituitary cells are activated by physiological elevations of the cytosolic Ca^{2+} concentration at the normal resting potential. *Acta Physiol Scand* **161,** 227-237.

Heginbotham L, Lu Z, Abramson T & MacKinnon R. (1994). Mutations in the K^+ channel signature sequence. *Biophys J* **66,** 1061-1067.

Hermann A & Gorman AL. (1981). Effects of tetraethylammonium on potassium currents in a molluscan neurons. *J Gen Physiol* **78,** 87-110.

Hoffman WE, Charbel FT, Gonzalez-Portillo G & Ausman JI. (1999). Measurement of ischemia by changes in tissue oxygen, carbon dioxide, and pH. *Surg Neurol* **51,** 654-658.

Hogg DS, Davies AR, McMurray G & Kozlowski RZ. (2002). K(V)2.1 channels mediate hypoxic inhibition of I(KV) in native pulmonary arterial smooth muscle cells of the rat. *Cardiovasc Res* **55,** 349-360.

Horrigan FT & Aldrich RW. (2002). Coupling between voltage sensor activation, Ca2+ binding and channel opening in large conductance (BK) potassium channels. *J Gen Physiol* **120,** 267-305.

Hu L, Yang H, Shi J & Cui J. (2006a). Effects of multiple metal binding sites on calcium and magnesium-dependent activation of BK channels. *J Gen Physiol* **127,** 35-49.

Hu L, Yang HH, Shi JY & Cui J. (2006b). Effects of multiple metal binding sites on calcium and magnesium-dependent activation of BK channels. *Journal of General Physiology* **127,** 35-49.

Hulme JT, Ahn M, Hauschka SD, Scheuer T & Catterall WA. (2002). A novel leucine zipper targets AKAP15 and cyclic AMP-dependent protein kinase to the C terminus of the skeletal muscle Ca^{2+} channel and modulates its function. *J Biol Chem* **277,** 4079-4087.

Jaggar JH, Porter VA, Lederer WJ & Nelson MT. (2000). Calcium sparks in smooth muscle. *Am J Physiol Cell Physiol* **278,** C235-256.

Jan LY & Jan YN. (1997). Voltage-gated and inwardly rectifying potassium channels. *J Physiol* **505 (Pt 2),** 267-282.

Jiang C & Haddad GG. (1994). A direct mechanism for sensing low oxygen levels by central neurons. *Proc Natl Acad Sci U S A* **91,** 7198-7201.

Jiang Y, Lee A, Chen J, Cadene M, Chait BT & MacKinnon R. (2002a). Crystal structure and mechanism of a calcium-gated potassium channel. *Nature* **417,** 515-522.

Jiang Y, Lee A, Chen J, Cadene M, Chait BT & MacKinnon R. (2002b). The open pore conformation of potassium channels. *Nature* **417,** 523-526.

Jiang Y, Pico A, Cadene M, Chait BT & MacKinnon R. (2001). Structure of the RCK domain from the E. coli K^+ channel and demonstration of its presence in the human BK channel. *Neuron* **29,** 593-601.

Jiang Z, Wallner M, Meera P & Toro L. (1999). Human and rodent MaxiK channel β-subunit genes: cloning and characterization. *Genomics* **55,** 57-67.

Johannes T, Mik EG & Ince C. (2006). Dual-wavelength phosphorimetry for determination of cortical and subcortical microvascular oxygenation in rat kidney. *J Appl Physiol* **100,** 1301-1310.

John VH, Dale TJ, Hollands EC, Chen MX, Partington L, Downie DL, Meadows HJ & Trezise DJ. (2007). Novel 384-well population patch clamp electrophysiology assays for Ca^{2+}-activated K+ channels. *J Biomol Screen* **12,** 50-60.

Johnson RP, O'Kelly IM & Fearon IM. (2004). System-specific O_2 sensitivity of the tandem pore domain K^+ channel TASK-1. *Am J Physiol Cell Physiol* **286,** C391-397.

Jovanovic S, Crawford RM, Ranki HJ & Jovanovic A. (2003). Large Conductance Ca^{2+}-Activated K^+ Channels Sense Acute Changes in Oxygen Tension in Alveolar Epithelial Cells. *Am J Respir Cell Mol Biol* **28,** 363-372.

Jurica MS & Moore MJ. (2003). Pre-mRNA splicing: awash in a sea of proteins. *Mol Cell* **12,** 5-14.

Kaczorowski GJ, Knaus HG, Leonard RJ, McManus OB & Garcia ML. (1996). High-conductance calcium-activated potassium channels; structure, pharmacology, and function. *J Bioenerg Biomembr* **28,** 255-267.

Kang J, Huguenard JR & Prince DA. (1996). Development of BK channels in neocortical pyramidal neurons. *J Neurophysiol* **76,** 188-198.

Katsuki H, Shinohara A, Fujimoto S, Kume T & Akaike A. (2005). Tetraethylammonium exacerbates ischemic neuronal injury in rat cerebrocortical slice cultures. *Eur J Pharmacol* **508,** 85-91.

Kauffman RF, Taylor RW & Pfeiffer DR. (1980). Cation transport and specificity of ionomycin. Comparison with ionophore A23187 in rat liver mitochondria. *J Biol Chem* **255,** 2735-2739.

Kemp PJ. (2006). Detecting acute changes in oxygen: will the real sensor please stand up? *Exp Physiol* **91,** 829-834.

Kim HJ, Lim HH, Rho SH, Eom SH & Park CS. (2006a). Hydrophobic interface between two regulators of K+ conductance domains critical for calcium-dependent activation of large conductance Ca^{2+}-activated K^+ channels. *J Biol Chem* **281,** 38573-38581.

Kim I, Kim JH & Carroll JL. (2006b). Postnatal changes in gene expression of subfamilies of TASK K^+ channels in rat carotid body. *Adv Exp Med Biol* **580,** 43-47; discussion 351-359.

Kline DD, Peng YJ, Manalo DJ, Semenza GL & Prabhakar NR. (2002). Defective carotid body function and impaired ventilatory responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1 alpha. *Proc Natl Acad Sci U S A* **99,** 821-826.

Knaus H-G, Eberhart A, Koch ROA, Munujos P, Schmalhofer WA, Warmke JW, Kaczorowski GJ & Garcia ML. (1995). Characterization of Tissue-expressed alpha Subunits of the High Conductance Ca^{2+}-activated K^+ Channel. *J Biol Chem* **270,** 22434-22439.

Knaus HG, Eberhart A, Glossmann H, Munujos P, Kaczorowski GJ & Garcia ML. (1994a). Pharmacology and structure of high conductance calcium-activated potassium channels. *Cell Signal* **6,** 861-870.

Knaus HG, McManus OB, Lee SH, Schmalhofer WA, Garcia-Calvo M, Helms LM, Sanchez M, Giangiacomo K, Reuben JP, Smith AB, 3rd & et al. (1994b). Tremorgenic indole alkaloids potently inhibit smooth muscle high-conductance calcium-activated potassium channels. *Biochemistry* **33,** 5819-5828.

Kobayashi H, Adachi-Akahane S & Nagao T. (2000). Involvement of BK(Ca) channels in the relaxation of detrusor muscle via β-adrenoceptors. *Eur J Pharmacol* **404,** 231-238.

Kornblihtt AR, de la Mata M, Fededa JP, Munoz MJ & Nogues G. (2004). Multiple links between transcription and splicing. *Rna* **10,** 1489-1498.

Koval OM, Fan Y & Rothberg BS. (2007). A role for the S0 transmembrane segment in voltage-dependent gating of BK channels. *J Gen Physiol* **129,** 209-220.

Kristian T & Siesjo BK. (1998). Calcium in ischemic cell death. *Stroke* **29,** 705-718.

Kurek I, Kawagoe Y, Jacob-Wilk D, Doblin M & Delmer D. (2002). Dimerization of cotton fiber cellulose synthase catalytic subunits occurs via oxidation of the zinc-binding domains. *Proc Natl Acad Sci U S A* **99,** 11109-11114.

Kwon SH & Guggino WB. (2004). Multiple sequences in the C terminus of MaxiK channels are involved in expression, movement to the cell surface, and apical localization. *Proc Natl Acad Sci U S A* **101,** 15237-15242.

Lagrutta A, Shen KZ, North RA & Adelman JP. (1994). Functional differences among alternatively spliced variants of Slowpoke, a Drosophila calcium-activated potassium channel. *J Biol Chem* **269,** 20347-20351.

Latorre R & Brauchi S. (2006). Large conductance Ca^{2+}-activated K^+ (BK) channel: activation by Ca^{2+} and voltage. *Biol Res* **39,** 385-401.

Lee K, Rowe IC & Ashford ML. (1995). NS 1619 activates BK_{Ca} channel activity in rat cortical neurones. *Eur J Pharmacol* **280,** 215-219.

Levitan IB. (1994). Modulation of ion channels by protein phosphorylation and dephosphorylation. *Annu Rev Physiol* **56,** 193-212.

Levitan IB. (1999). Modulation of ion channels by protein phosphorylation. How the brain works. *Adv Second Messenger Phosphoprotein Res* **33,** 3-22.

Levitan IB. (2006). Signaling protein complexes associated with neuronal ion channels. *Nat Neurosci* **9,** 305-310.

Lewis A, Hartness ME, Chapman CG, Fearon IM, Meadows HJ, Peers C & Kemp PJ. (2001). Recombinant hTASK1 is an O_2-sensitive K^+ channel. *Biochem Biophys Res Commun* **285,** 1290-1294.

Lewis A, Peers C, Ashford ML & Kemp PJ. (2002). Hypoxia inhibits human recombinant large conductance, Ca^{2+}-activated K^+ (maxi-K) channels by a mechanism which is membrane delimited and Ca(2+) sensitive. *J Physiol* **540,** 771-780.

Li W & Aldrich RW. (2004). Unique inner pore properties of BK channels revealed by quaternary ammonium block. *J Gen Physiol* **124,** 43-57.

Ling S, Sheng JZ & Braun AP. (2004). The calcium-dependent activity of large-conductance, calcium-activated K^+ channels is enhanced by Pyk2- and Hck-induced tyrosine phosphorylation. *Am J Physiol Cell Physiol* **287,** C698-706.

Lingle CJ. (2007). Gating rings formed by RCK domains: keys to gate opening. *J Gen Physiol* **129,** 101-107.

Liu G, Shi J, Yang L, Cao L, Park SM, Cui J & Marx SO. (2004). Assembly of a Ca^{2+}-dependent BK channel signaling complex by binding to β2 adrenergic receptor. *Embo J* **23,** 2196-2205.

Liu H, Moczydlowski E & Haddad GG. (1999). O_2 deprivation inhibits Ca^{2+}-activated K^+ channels via cytosolic factors in mice neocortical neurons. *J Clin Invest* **104,** 577-588.

Liu X, Chang Y, Reinhart PH, Sontheimer H & Chang Y. (2002). Cloning and characterization of glioma BK, a novel BK channel isoform highly expressed in human glioma cells. *J Neurosci* **22,** 1840-1849.

Liu Y, Pleyte K, Knaus HG & Rusch NJ. (1997). Increased expression of Ca^{2+}-sensitive K^+ channels in aorta of hypertensive rats. *Hypertension* **30,** 1403-1409.

Loane DJ, Lima PA & Marrion NV. (2007). Co-assembly of N-type Ca^{2+} and BK channels underlies functional coupling in rat brain. *J Cell Sci* **120,** 985-995.

Lopez-Barneo J, del Toro R, Levitsky KL, Chiara MD & Ortega-Saenz P. (2004). Regulation of oxygen sensing by ion channels. *J Appl Physiol* **96,** 1187-1195; discussion 1170-1182.

Lopez-Barneo J, Lopez-Lopez JR, Urena J & Gonzalez C. (1988). Chemotransduction in the carotid body: K^+ current modulated by PO2 in type I chemoreceptor cells. *Science* **241,** 580-582.

Lopez-Barneo J, Ortega-Saenz P, Molina A, Franco-Obregon A, Urena J & Castellano A. (1997). Oxygen sensing by ion channels. *Kidney Int* **51,** 454-461.

Lopez-Barneo J, Pardal R & Ortega-Saenz P. (2001). Cellular mechanism of oxygen sensing. *Annu Rev Physiol* **63,** 259-287.

Lu R, Alioua A, Kumar Y, Eghbali M, Stefani E & Toro L. (2006). MaxiK channel partners: physiological impact. *J Physiol* **570,** 65-72.

Lua BL & Low BC. (2005). Cortactin phosphorylation as a switch for actin cytoskeletal network and cell dynamics control. *FEBS Lett* **579,** 577-585.

MacDonald SH, Ruth P, Knaus HG & Shipston MJ. (2006). Increased large conductance calcium-activated potassium (BK) channel expression accompanied by STREX variant downregulation in the developing mouse CNS. *BMC Dev Biol* **6,** 37.

MacKinnon R. (2003). Potassium channels. *FEBS Lett* **555,** 62-65.

Marijic J, Li Q, Song M, Nishimaru K, Stefani E & Toro L. (2001). Decreased expression of voltage- and $Ca(^{2+})$-activated $K(^{+})$ channels in coronary smooth muscle during aging. *Circ Res* **88,** 210-216.

Masson N & Ratcliffe PJ. (2003). HIF prolyl and asparaginyl hydroxylases in the biological response to intracellular $O(_2)$ levels. *J Cell Sci* **116,** 3041-3049.

Mathialahan T, Maclennan KA, Sandle LN, Verbeke C & Sandle GI. (2005). Enhanced large intestinal potassium permeability in end-stage renal disease. *J Pathol* **206,** 46-51.

McCartney CE, McClafferty H, Huibant JM, Rowan EG, Shipston MJ & Rowe IC. (2005). A cysteine-rich motif confers hypoxia sensitivity to mammalian large conductance voltage- and Ca-activated K (BK) channel α-subunits. *Proc Natl Acad Sci U S A* **102,** 17870-17876.

McCobb DP, Hara Y, Lai GJ, Mahmoud SF & Flugge G. (2003). Subordination stress alters alternative splicing of the Slo gene in tree shrew adrenals. *Horm Behav* **43,** 180-186.

McManus OB, Helms LM, Pallanck L, Ganetzky B, Swanson R & Leonard RJ. (1995). Functional role of the beta subunit of high conductance calcium-activated potassium channels. *Neuron* **14,** 645-650.

Meera P, Wallner M, Song M & Toro L. (1997). Large conductance voltage- and calcium-dependent K^+ channel, a distinct member of voltage-dependent ion channels with seven N-terminal transmembrane segments (S0-S6), an extracellular N terminus, and an intracellular (S9-S10) C terminus. *Proc Natl Acad Sci U S A* **94,** 14066-14071.

Meera P, Wallner M & Toro L. (2000). A neuronal beta subunit (KCNMB4) makes the large conductance, voltage- and Ca^{2+}-activated K^+ channel resistant to charybdotoxin and iberiotoxin. *Proc Natl Acad Sci U S A* **97,** 5562-5567.

Meredith AL, Thorneloe KS, Werner ME, Nelson MT & Aldrich RW. (2004). Overactive bladder and incontinence in the absence of the BK large conductance Ca^{2+}-activated K^+ channel. *J Biol Chem* **279,** 36746-36752.

Miller C, Moczydlowski E, Latorre R & Phillips M. (1985). Charybdotoxin, a protein inhibitor of single Ca^{2+}-activated K^+ channels from mammalian skeletal muscle. *Nature* **313,** 316-318.

Morimoto T, Sakamoto K, Sade H, Ohya S, Muraki K & Imaizumi Y. (2007). Voltage-sensitive oxonol dyes are novel large-conductance Ca^{2+}-activated

K^+ channel activators selective for beta1 and beta4 but not for β2 subunits. *Mol Pharmacol* **71,** 1075-1088.

Morrow JP, Zakharov SI, Liu G, Yang L, Sok AJ & Marx SO. (2006). Defining the BK channel domains required for β1-subunit modulation. *Proc Natl Acad Sci U S A* **103,** 5096-5101.

Nara M, Dhulipala PD, Wang YX & Kotlikoff MI. (1998). Reconstitution of β-adrenergic modulation of large conductance, calcium-activated potassium (maxi-K) channels in Xenopus oocytes. Identification of the camp-dependent protein kinase phosphorylation site. *J Biol Chem* **273,** 14920-14924.

Neher E & Sakmann B. (1976). Single-channel currents recorded from membrane of denervated frog muscle fibres. *Nature* **260,** 799-802.

Nelson MT. (1992). Regulation of arterial tone by potassium channels. *Jpn J Pharmacol* **58 Suppl 2,** 238P-242P.

Nelson MT & Bonev AD. (2004). The beta1 subunit of the Ca^{2+}-sensitive K^+ channel protects against hypertension. *J Clin Invest* **113,** 955-957.

Nichols CG. (2006). K_{ATP} channels as molecular sensors of cellular metabolism. *Nature* **440,** 470-476.

Nimigean CM & Magleby KL. (1999). The beta subunit increases the Ca^{2+} sensitivity of large conductance Ca^{2+}-activated potassium channels by retaining the gating in the bursting states. *J Gen Physiol* **113,** 425-440.

Nishimaru K, Eghbali M, Lu R, Marijic J, Stefani E & Toro L. (2004). Functional and molecular evidence of MaxiK channel β1 subunit decrease with coronary artery ageing in the rat. *J Physiol* **559,** 849-862.

Niu X & Magleby KL. (2002). Stepwise contribution of each subunit to the cooperative activation of BK channels by Ca^{2+}. *Proc Natl Acad Sci U S A* **99,** 11441-11446.

O'Kelly I, Stephens RH, Peers C & Kemp PJ. (1999). Potential identification of the O_2-sensitive K^+ current in a human neuroepithelial body-derived cell line. *Am J Physiol* **276,** L96-L104.

Olesen SP, Munch E, Moldt P & Drejer J. (1994). Selective activation of $Ca^{(2+)}$-dependent K^+ channels by novel benzimidazolone. *Eur J Pharmacol* **251,** 53-59.

Orio P, Rojas P, Ferreira G & Latorre R. (2002). New disguises for an old channel: MaxiK channel beta-subunits. *News Physiol Sci* **17,** 156-161.

Ortega-Saenz P, Garcia-Fernandez M, Pardal R, Alvarez E & Lopez-Barneo J. (2003). Studies on glomus cell sensitivity to hypoxia in carotid body slices. *Adv Exp Med Biol* **536,** 65-73.

Ortega-Saenz P, Pascual A, Gomez-Diaz R & Lopez-Barneo J. (2006). Acute oxygen sensing in heme oxygenase-2 null mice. *J Gen Physiol* **128,** 405-411.

Ortega-Saenz P, Pascual A, Piruat JI & Lopez-Barneo J. (2007). Mechanisms of acute oxygen sensing by the carotid body: lessons from genetically modified animals. *Respir Physiol Neurobiol* **157,** 140-147.

Papazian DM, Timpe LC, Jan YN & Jan LY. (1991). Alteration of voltage-dependence of Shaker potassium channel by mutations in the S4 sequence. *Nature* **349,** 305-310.

Pardal R & Lopez-Barneo J. (2002). Carotid body thin slices: responses of glomus cells to hypoxia and $K^{(+)}$-channel blockers. *Respir Physiol Neurobiol* **132,** 69-79.

Pardal R, Ludewig U, Garcia-Hirschfeld J & Lopez-Barneo J. (2000). Secretory responses of intact glomus cells in thin slices of rat carotid body to hypoxia and tetraethylammonium. *Proc Natl Acad Sci U S A* **97,** 2361-2366.

Park JS, Wang M, Park SJ & Lee SH. (1999). Zinc finger of replication protein A, a non-DNA binding element, regulates its DNA binding activity through redox. *J Biol Chem* **274,** 29075-29080.

Park MK, Lee SH, Lee SJ, Ho WK & Earm YE. (1995). Different modulation of Ca-activated K channels by the intracellular redox potential in pulmonary and ear arterial smooth muscle cells of the rabbit. *Pflugers Arch* **430,** 308-314.

Pearson RB & Kemp BE. (1991). Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. *Methods Enzymol* **200,** 62-81.

Pechlivanis M & Kuhlmann J. (2006). Hydrophobic modifications of Ras proteins by isoprenoid groups and fatty acids--More than just membrane anchoring. *Biochim Biophys Acta* **1764,** 1914-1931.

Peers C. (1990). Hypoxic suppression of K+ currents in type I carotid body cells: selective effect on the Ca^{2+}-activated K^+ current. *Neurosci Lett* **119,** 253-256.

Peers C & Kemp PJ. (2004). Ion channel regulation by chronic hypoxia in models of acute oxygen sensing. *Cell Calcium* **36,** 341-348.

Perez-Garcia MT, Colinas O, Miguel-Velado E, Moreno-Dominguez A & Lopez-Lopez JR. (2004). Characterization of the Kv channels of mouse carotid body chemoreceptor cells and their role in oxygen sensing. *J Physiol* **557,** 457-471.

Perez G & Toro L. (1994). Differential modulation of large-conductance KCa channels by PKA in pregnant and nonpregnant myometrium. *Am J Physiol* **266,** C1459-1463.

Petkov GV, Bonev AD, Heppner TJ, Brenner R, Aldrich RW & Nelson MT. (2001). β1-subunit of the Ca^{2+}-activated K^+ channel regulates contractile activity of mouse urinary bladder smooth muscle. *J Physiol* **537,** 443-452.

Petkov GV & Nelson MT. (2005). Differential regulation of Ca^{2+}-activated K^+ channels by beta-adrenoceptors in guinea pig urinary bladder smooth muscle. *Am J Physiol Cell Physiol* **288,** C1255-1263.

Piskorowski R & Aldrich RW. (2002). Calcium activation of BK_{Ca} potassium channels lacking the calcium bowl and RCK domains. *Nature* **420,** 499-502.

Pluznick JL & Sansom SC. (2006). BK channels in the kidney: role in K(+) secretion and localization of molecular components. *Am J Physiol Renal Physiol* **291,** F517-529.

Pluznick JL, Wei P, Carmines PK & Sansom SC. (2003). Renal fluid and electrolyte handling in BK_{Ca}-β1-/- mice. *Am J Physiol Renal Physiol* **284,** F1274-1279.

Porter VA, Rhodes MT, Reeve HL & Cornfield DN. (2001). Oxygen-induced fetal pulmonary vasodilation is mediated by intracellular calcium activation of $K_{(Ca)}$ channels. *Am J Physiol Lung Cell Mol Physiol* **281,** L1379-1385.

Qian X, Niu X & Magleby KL. (2006). Intra- and intersubunit cooperativity in activation of BK channels by Ca^{2+}. *J Gen Physiol* **128,** 389-404.

Quirk JC & Reinhart PH. (2001). Identification of a novel tetramerization domain in large conductance $K_{(Ca)}$ channels. *Neuron* **32,** 13-23.

Ramanathan K, Michael TH, Jiang GJ, Hiel H & Fuchs PA. (1999). A molecular mechanism for electrical tuning of cochlear hair cells. *Science* **283,** 215-217.

Reinhart PH & Levitan IB. (1995). Kinase and phosphatase activities intimately associated with a reconstituted calcium-dependent potassium channel. *J Neurosci* **15,** 4572-4579.

Resnik E, Herron J, Fu R, Ivy DD & Cornfield DN. (2006). Oxygen tension modulates the expression of pulmonary vascular BK_{Ca} channel α- and β-subunits. *Am J Physiol Lung Cell Mol Physiol* **290,** L761-L768.

Ribeiro CM, McKay RR, Hosoki E, Bird GS & Putney JW, Jr. (2000). Effects of elevated cytoplasmic calcium and protein kinase C on endoplasmic reticulum structure and function in HEK293 cells. *Cell Calcium* **27,** 175-185.

Rieg T, Vallon V, Sausbier M, Sausbier U, Kaissling B, Ruth P & Osswald H. (2007). The role of the BK channel in potassium homeostasis and flow-induced renal potassium excretion. *Kidney Int* **72,** 566-573.

Riesco-Fagundo AM, Perez-Garcia MT, Gonzalez C & Lopez-Lopez JR. (2001). $O(2)$ modulates large-conductance $Ca(^{2+})$-dependent $K(^{+})$ channels of rat chemoreceptor cells by a membrane-restricted and CO-sensitive mechanism. *Circ Res* **89,** 430-436.

Robitaille R, Garcia ML, Kaczorowski GJ & Charlton MP. (1993). Functional colocalization of calcium and calcium-gated potassium channels in control of transmitter release. *Neuron* **11,** 645-655.

Roosild TP, Le KT & Choe S. (2004). Cytoplasmic gatekeepers of K^+-channel flux: a structural perspective. *Trends Biochem Sci* **29,** 39-45.

Runden-Pran E, Haug FM, Storm JF & Ottersen OP. (2002). BK channel activity determines the extent of cell degeneration after oxygen and glucose deprivation: a study in organotypical hippocampal slice cultures. *Neuroscience* **112,** 277-288.

Rundfeldt C. (1999). Characterization of the K^+ channel opening effect of the anticonvulsant retigabine in PC12 cells. *Epilepsy Res* **35,** 99-107.

Ruttiger L, Sausbier M, Zimmermann U, Winter H, Braig C, Engel J, Knirsch M, Arntz C, Langer P, Hirt B, Muller M, Kopschall I, Pfister M, Munkner S, Rohbock K, Pfaff I, Rusch A, Ruth P & Knipper M. (2004). Deletion of the Ca^{2+}-activated potassium (BK) α-subunit but not the BKβ1-subunit leads to progressive hearing loss. *Proc Natl Acad Sci U S A* **101,** 12922-12927.

Rychkov GY, Adams MB, McMillen IC & Roberts ML. (1998). Oxygen-sensing mechanisms are present in the chromaffin cells of the sheep adrenal medulla before birth. *J Physiol* **509 (Pt 3),** 887-893.

Sah P. (1996). $Ca(^{2+})$-activated K^+ currents in neurones: types, physiological roles and modulation. *Trends Neurosci* **19,** 150-154.

Saikumar P, Dong Z, Patel Y, Hall K, Hopfer U, Weinberg JM & Venkatachalam MA. (1998). Role of hypoxia-induced Bax translocation and cytochrome c release in reoxygenation injury. *Oncogene* **17,** 3401-3415.

Saito M, Nelson C, Salkoff L & Lingle CJ. (1997). A cysteine-rich domain defined by a novel exon in a slo variant in rat adrenal chromaffin cells and PC12 cells. *J Biol Chem* **272,** 11710-11717.

Saleem F, Rowe I & Shipston M. (2007). Identification of amino acid residues that influence both the calcium and hypoxia sensitivity of the STREX splice variant of BK channel α-subunits. In *Society for Neuroscience, Anuual Meeting 2007*. San Diego, USA.

Salkoff L, Butler A, Ferreira G, Santi C & Wei A. (2006). High-conductance potassium channels of the SLO family. *Nat Rev Neurosci* **7,** 921-931.

Salmeen A, Andersen JN, Myers MP, Meng TC, Hinks JA, Tonks NK & Barford D. (2003). Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-amide intermediate. *Nature* **423,** 769-773.

Sanchez D, Lopez-Lopez JR, Perez-Garcia MT, Sanz-Alfayate G, Obeso A, Ganfornina MD & Gonzalez C. (2002). Molecular identification of Kvalpha subunits that contribute to the oxygen-sensitive K^+ current of chemoreceptor cells of the rabbit carotid body. *J Physiol* **542,** 369-382.

Sanchez M & McManus OB. (1996). Paxilline inhibition of the alpha-subunit of the high-conductance calcium-activated potassium channel. *Neuropharmacology* **35,** 963-968.

Sausbier M, Arntz C, Bucurenciu I, Zhao H, Zhou XB, Sausbier U, Feil S, Kamm S, Essin K, Sailer CA, Abdullah U, Krippeit-Drews P, Feil R, Hofmann F, Knaus HG, Kenyon C, Shipston MJ, Storm JF, Neuhuber W, Korth M, Schubert R, Gollasch M & Ruth P. (2005). Elevated blood pressure linked to primary hyperaldosteronism and impaired vasodilation in BK channel-deficient mice. *Circulation* **112,** 60-68.

Sausbier M, Hu H, Arntz C, Feil S, Kamm S, Adelsberger H, Sausbier U, Sailer CA, Feil R, Hofmann F, Korth M, Shipston MJ, Knaus HG, Wolfer DP, Pedroarena CM, Storm JF & Ruth P. (2004). Cerebellar ataxia and Purkinje cell dysfunction caused by Ca2+- activated K^+ channel deficiency. *Procs Natl Academy of Sciences* **101,** 9474-9478.

Schmalhofer WA, Sanchez M, Dai G, Dewan A, Secades L, Hanner M, Knaus HG, McManus OB, Kohler M, Kaczorowski GJ & Garcia ML. (2005). Role of the C-terminus of the high-conductance calcium-activated potassium channel in channel structure and function. *Biochemistry* **44,** 10135-10144.

Schreiber M, Yuan A & Salkoff L. (1999). Transplantable sites confer calcium sensitivity to BK channels. *Nat Neurosci* **2,** 416-421.

Schubert R & Nelson MT. (2001). Protein kinases: tuners of the BK_{Ca} channel in smooth muscle. *Trends Pharmacol Sci* **22,** 505-512.

Schumacher M & Adelman JP. (2002). Ion channels: an open and shut case. *Nature* **417,** 501-502.

Sellers AJ & Ashford ML. (1994). Activation of BK_{Ca} channels in acutely dissociated neurones from the rat ventromedial hypothalamus by NS 1619. *Br J Pharmacol* **113,** 659-661.

Shapiro HM. (2000). Membrane potential estimation by flow cytometry. *Methods* **21,** 271-279.

Shen KZ, Lagrutta A, Davies NW, Standen NB, Adelman JP & North RA. (1994). Tetraethylammonium block of Slowpoke calcium-activated potassium channels expressed in Xenopus oocytes: evidence for tetrameric channel formation. *Pflugers Arch* **426,** 440-445.

Shi J & Cui J. (2001). Intracellular $Mg^{(2+)}$ enhances the function of BK-type $Ca^{(2+)}$-activated $K^{(+)}$ channels. *J Gen Physiol* **118,** 589-606.

Shi J, Krishnamoorthy G, Yang Y, Hu L, Chaturvedi N, Harilal D, Qin J & Cui J. (2002). Mechanism of magnesium activation of calcium-activated potassium channels. *Nature* **418,** 876-880.

Shimoda LA, Manalo DJ, Sham JS, Semenza GL & Sylvester JT. (2001). Partial HIF-1α deficiency impairs pulmonary arterial myocyte electrophysiological responses to hypoxia. *Am J Physiol Lung Cell Mol Physiol* **281,** L202-208.

Shipston MJ. (2001). Alternative splicing of potassium channels: a dynamic switch of cellular excitability. *Trends in Cell Biology* **11,** 353-358.

Strang C, Cushman SJ, DeRubeis D, Peterson D & Pfaffinger PJ. (2001). A central role for the T1 domain in voltage-gated potassium channel formation and function. *J Biol Chem* **276,** 28493-28502.

Tang XD, Daggett H, Hanner M, Garcia ML, McManus OB, Brot N, Weissbach H, Heinemann SH & Hoshi T. (2001). Oxidative regulation of large conductance calcium-activated potassium channels. *J Gen Physiol* **117,** 253-274.

Tang XD, Garcia ML, Heinemann SH & Hoshi T. (2004). Reactive oxygen species impair Slo1 BK channel function by altering cysteine-mediated calcium sensing. *Nat Struct Mol Biol* **11,** 171-178.

Thomas P & Smart TG. (2005). HEK293 cell line: a vehicle for the expression of recombinant proteins. *J Pharmacol Toxicol Methods* **51,** 187-200.

Thompson RJ, Jackson A & Nurse CA. (1997). Developmental loss of hypoxic chemosensitivity in rat adrenomedullary chromaffin cells. *J Physiol* **498 (Pt 2),** 503-510.

Tian L, Chen L, McClafferty H, Sailer CA, Ruth P, Knaus HG & Shipston MJ. (2006). A noncanonical SH3 domain binding motif links BK channels to the actin cytoskeleton via the SH3 adapter cortactin. *Faseb J* **20,** 2588-2590.

Tian L, Coghill LS, McClafferty H, MacDonald SHF, Antoni FA, Ruth P, Knaus HG & Shipston MJ. (2004). Distinct stoichiometry of BK_{Ca} channel tetrarner phosphorylation specifies channel activation and inhibition by cAMP-dependent protein kinase. *Proc Natl Academy of Sciences* **101,** 11897-11902.

Tian L, Duncan RR, Hammond MS, Coghill LS, Wen H, Rusinova R, Clark AG, Levitan IB & Shipston MJ. (2001a). Alternative splicing switches potassium channel sensitivity to protein phosphorylation. *J Biol Chem* **276,** 7717-7720.

Tian L, McClafferty H, Chen L & Shipston MJ. (2007). Reversible tyrosine protein phosphorylation regulates large conductance voltage- and calcium- activated potassium (BK) channels via cortactin. *J Biol Chem.*

Tian L, McClafferty H, Chen L & Shipston MJ. (2008). Reversible Tyrosine Protein Phosphorylation Regulates Large Conductance Voltage- and Calcium-activated Potassium Channels via Cortactin. *J Biol Chem* **283,** 3067-3076.

Tian LJ, Coghill LS, MacDonald SHF, Armstrong DL & Shipston MJ. (2003). Leucine zipper domain targets cAMP-dependent protein kinase to mammalian BK channels. *J Biol Chem* **278,** 8669-8677.

Tian LJ, Hammond MSL, Florance H, Antoni FA & Shipston MJ. (2001b). Alternative splicing determines sensitivity of murine calcium- activated potassium channels to glucocorticoids. *J Physiol* **537,** 57-68.

Toro L, Wallner M, Meera P & Tanaka Y. (1998). Maxi-K($_{Ca}$), a Unique Member of the Voltage-Gated K Channel Superfamily. *News Physiol Sci* **13,** 112-117.

Torres YP, Morera FJ, Carvacho I & Latorre R. (2007). A marriage of convenience: beta-subunits and voltage-dependent K+ channels. *J Biol Chem* **282,** 24485-24489.

Tricarico D, Capriulo R & Camerino DC. (2002). Involvement of K(Ca^{2+}) channels in the local abnormalities and hyperkalemia following the ischemia-reperfusion injury of rat skeletal muscle. *Neuromuscul Disord* **12,** 258-265.

Tseng-Crank J, Foster CD, Krause JD, Mertz R, Godinot N, DiChiara TJ & Reinhart PH. (1994). Cloning, expression, and distribution of functionally distinct Ca($^{2+}$)-activated K^+ channel isoforms from human brain. *Neuron* **13,** 1315-1330.

Turner MS, Haywood GA, Andreka P, You L, Martin PE, Evans WH, Webster KA & Bishopric NH. (2004). Reversible connexin 43 dephosphorylation

during hypoxia and reoxygenation is linked to cellular ATP levels. *Circ Res* **95,** 726-733.

Urena J, Fernandez-Chacon R, Benot AR, Alvarez de Toledo GA & Lopez-Barneo J. (1994). Hypoxia induces voltage-dependent Ca^{2+} entry and quantal dopamine secretion in carotid body glomus cells. *Proc Natl Acad Sci* **91,** 10208-10211.

van Montfort RL, Congreve M, Tisi D, Carr R & Jhoti H. (2003). Oxidation state of the active-site cysteine in protein tyrosine phosphatase 1B. *Nature* **423,** 773-777.

Vergara C, Latorre R, Marrion NV & Adelman JP. (1998). Calcium-activated potassium channels. *Curr Opin Neurobiol* **8,** 321-329.

Wallner M, Meera P, Ottolia M, Kaczorowski GJ, Latorre R, Garcia ML, Stefani E & Toro L. (1995). Characterization of and modulation by a beta-subunit of a human maxi K_{Ca} channel cloned from myometrium. *Receptors Channels* **3,** 185-199.

Wallner M, Meera P & Toro L. (1996). Determinant for β-subunit regulation in high-conductance voltage-activated and $Ca^{(2+)}$-sensitive K^+ channels: an additional transmembrane region at the N terminus. *Proc Natl Acad Sci U S A* **93,** 14922-14927.

Wallner M, Meera P & Toro L. (1999). Molecular basis of fast inactivation in voltage and Ca^{2+}-activated K^+ channels: a transmembrane beta-subunit homolog. *Proc Natl Acad Sci U S A* **96,** 4137-4142.

Wang SX, Ikeda M & Guggino WB. (2003). The cytoplasmic tail of large conductance, voltage- and Ca^{2+}-activated K^+ (MaxiK) channel is necessary for its cell surface expression. *J Biol Chem* **278,** 2713-2722.

Wanner SG, Koch RO, Koschak A, Trieb M, Garcia ML, Kaczorowski GJ & Knaus HG. (1999). High-conductance calcium-activated potassium channels in rat brain: pharmacology, distribution, and subunit composition. *Biochemistry* **38,** 5392-5400.

Ward JP. (2008). Oxygen sensors in context. *Biochim Biophys Acta* **1777,** 1-14.

Weaver AK, Bomben VC & Sontheimer H. (2006). Expression and function of calcium-activated potassium channels in human glioma cells. *Glia* **54,** 223-233.

Weaver AK, Liu X & Sontheimer H. (2004). Role for calcium-activated potassium channels (BK) in growth control of human malignant glioma cells. *J Neurosci Res* **78,** 224-234.

Wei A, Solaro C, Lingle C & Salkoff L. (1994). Calcium sensitivity of BK-type K_{Ca} channels determined by a separable domain. *Neuron* **13,** 671-681.

Weiger TM, Langer T & Hermann A. (1998). External action of di- and polyamines on maxi calcium-activated potassium channels: an electrophysiological and molecular modeling study. *Biophys J* **74,** 722-730.

Weir EK, Lopez-Barneo J, Buckler KJ & Archer SL. (2005). Acute oxygen-sensing mechanisms. *N Engl J Med* **353,** 2042-2055.

Wellman GC & Nelson MT. (2003). Signaling between SR and plasmalemma in smooth muscle: sparks and the activation of Ca^{2+}-sensitive ion channels. *Cell Calcium* **34,** 211-229.

Whiteaker KL, Gopalakrishnan SM, Groebe D, Shieh CC, Warrior U, Burns DJ, Coghlan MJ, Scott VE & Gopalakrishnan M. (2001). Validation of FLIPR membrane potential dye for high throughput screening of potassium channel modulators. *J Biomol Screen* **6,** 305-312.

Widmer HA, Rowe ICM & Shipston MJ. (2003). Conditional protein phosphorylation regulates BK channel activity in rat cerebellar Purkinje neurons. *Journal of Physiology-London* **552,** 379-391.

Williams BA & Buckler KJ. (2004). Biophysical properties and metabolic regulation of a TASK-like potassium channel in rat carotid body type 1 cells. *Am J Physiol Lung Cell Mol Physiol* **286,** L221-230.

Williams SE, Wootton P, Mason HS, Bould J, Iles DE, Riccardi D, Peers C & Kemp PJ. (2004). Hemoxygenase-2 is an oxygen sensor for a calcium-sensitive potassium channel. *Science* **306,** 2093-2097.

Woda CB, Bragin A, Kleyman TR & Satlin LM. (2001). Flow-dependent K^+ secretion in the cortical collecting duct is mediated by a maxi-K channel. *Am J Physiol Renal Physiol* **280,** F786-793.

Wu SN, Lin MW & Wang YJ. (2008). Stimulatory actions of di-8-butyl-amino-naphthyl-ethylene-pyridinium-propyl-sulfonate (di-8-ANEPPS), voltage-sensitive dye, on the $BK_{(Ca)}$ channel in pituitary tumor (GH (3)) cells. *Pflugers Arch* **455,** 687-699.

Wyatt CN, Mustard KJ, Pearson SA, Dallas ML, Atkinson L, Kumar P, Peers C, Hardie DG & Evans AM. (2007). AMP-activated protein kinase mediates carotid body excitation by hypoxia. *J Biol Chem* **282,** 8092-8098.

Wyatt CN & Peers C. (1995). $Ca^{(2+)}$-activated K^+ channels in isolated type I cells of the neonatal rat carotid body. *J Physiol* **483 (Pt 3),** 559-565.

Wyatt CN, Wright C, Bee D & Peers C. (1995). O_2-sensitive K^+ currents in carotid body chemoreceptor cells from normoxic and chronically hypoxic rats and their roles in hypoxic chemotransduction. *Proc Natl Acad Sci U S A* **92,** 295-299.

Xia XM, Ding JP & Lingle CJ. (1999). Molecular basis for the inactivation of Ca^{2+}- and voltage-dependent BK channels in adrenal chromaffin cells and rat insulinoma tumor cells. *J Neurosci* **19,** 5255-5264.

Xia XM, Ding JP, Zeng XH, Duan KL & Lingle CJ. (2000). Rectification and rapid activation at low Ca^{2+} of Ca^{2+}-activated, voltage-dependent BK currents: consequences of rapid inactivation by a novel beta subunit. *J Neurosci* **20,** 4890-4903.

Xia XM, Zeng X & Lingle CJ. (2002). Multiple regulatory sites in large-conductance calcium-activated potassium channels. *Nature* **418,** 880-884.

Xie J & Black DL. (2001). A CaMK IV responsive RNA element mediates depolarization-induced alternative splicing of ion channels. *Nature* **410,** 936-939.

Xie J & McCobb DP. (1998). Control of alternative splicing of potassium channels by stress hormones. *Science* **280,** 443-446.

Xu W, Liu Y, Wang S, McDonald T, Van Eyk JE, Sidor A & O'Rourke B. (2002). Cytoprotective role of Ca^{2+}- activated K^+ channels in the cardiac inner mitochondrial membrane. *Science* **298,** 1029-1033.

Yamada A, Gaja N, Ohya S, Muraki K, Narita H, Ohwada T & Imaizumi Y. (2001). Usefulness and limitation of DiBAC4(3), a voltage-sensitive fluorescent dye, for the measurement of membrane potentials regulated by recombinant large conductance Ca^{2+}-activated K^+ channels in HEK293 cells. *Jpn J Pharmacol* **86,** 342-350.

Yamamoto Y, Kummer W, Atoji Y & Suzuki Y. (2002). TASK-1, TASK-2, TASK-3 and TRAAK immunoreactivities in the rat carotid body. *Brain Res* **950,** 304-307.

Yamamoto Y & Taniguchi K. (2006). Immunolocalization of tandem pore domain K^+ channels in the rat carotid body. *Adv Exp Med Biol* **580,** 9-14; discussion 351-359.

Yang H, Hu L, Shi J & Cui J. (2006). Tuning magnesium sensitivity of BK channels by mutations. *Biophys J* **91,** 2892-2900.

Yusifov T, Savalli N, Gandhi CS, Ottolia M & Olcese R. (2008). The RCK2 domain of the human BK_{Ca} channel is a calcium sensor. *Proc Natl Acad Sci U S A* **105,** 376-381.

Zarei MM, Zhu N, Alioua A, Eghbali M, Stefani E & Toro L. (2001). A novel MaxiK splice variant exhibits dominant-negative properties for surface expression. *J Biol Chem* **276,** 16232-16239.

Zeng XH, Xia XM & Lingle CJ. (2005). Divalent cation sensitivity of BK channel activation supports the existence of three distinct binding sites. *J Gen Physiol* **125,** 273-286.

Zhang G & Horrigan FT. (2005). Cysteine modification alters voltage- and $Ca^{(2+)}$-dependent gating of large conductance (BK) potassium channels. *J Gen Physiol* **125,** 213-236.

Zhang J, Davidson RM, Wei MD & Loew LM. (1998). Membrane electric properties by combined patch clamp and fluorescence ratio imaging in single neurons. *Biophys J* **74,** 48-53.

Zhang JH, Chung TD & Oldenburg KR. (1999). A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen* **4,** 67-73.

Zhang X, Solaro CR & Lingle CJ. (2001). Allosteric regulation of BK channel gating by $Ca^{(2+)}$ and $Mg^{(2+)}$ through a nonselective, low affinity divalent cation site. *J Gen Physiol* **118,** 607-636.

Zhang Y, Joiner WJ, Bhattacharjee A, Rassendren F, Magoski NS & Kaczmarek LK. (2004). The appearance of a protein kinase A-regulated splice isoform of slo is associated with the maturation of neurons that control reproductive behavior. *J Biol Chem* **279,** 52324-52330.

Zhang Y, Niu X, Brelidze TI & Magleby KL. (2006). Ring of negative charge in BK channels facilitates block by intracellular Mg^{2+} and polyamines through electrostatics. *J Gen Physiol* **128,** 185-202.

Zhorov BS & Tikhonov DB. (2004). Potassium, sodium, calcium and glutamate-gated channels: pore architecture and ligand action. *J Neurochem* **88,** 782-799.

Zhou XB, Arntz C, Kamm S, Motejlek K, Sausbier U, Wang GX, Ruth P & Korth M. (2001). A molecular switch for specific stimulation of the BK_{Ca} channel by cGMP and cAMP kinase. *J Biol Chem* **276,** 43239-43245.

Zhou XB, Wang GX, Huneke B, Wieland T & Korth M. (2000). Pregnancy switches adrenergic signal transduction in rat and human uterine myocytes as probed by BK_{Ca} channel activity. *J Physiol* **524 Pt 2,** 339-352.